Reverse transcriptase inhibitors suppress telomerase function and induce senescence-like processes in cultured mouse fibroblasts

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Abstract Spontaneous transformation of mouse embryonic fibroblasts in the presence of the reverse transcriptase inhibitors azidothymidine and carbovir led to the formation of telomerase-free clones. After prolonged cultivation of fibroblasts in the presence of carbovir, resistant cells with a very high level of telomerase activity were obtained. Azidothymidine and carbovir, but not dideoxycytidine, induced senescence-like processes in cultures of immortal mouse fibroblasts. After long-term incubation, cell proliferation gradually decreased, their morphology becoming similar to that of the senescent ones. The process was reversible: after inhibitor removal, the cells, including the giant ones, entered mitoses. All these data suggest that reverse transcriptase inhibitors block telomerase function in mouse cells.

Key words: Telomerase; Spontaneous transformation; Reverse transcriptase inhibitor; Senescence; (Mouse fibroblast)

1. Introduction

Telomeres of vertebrate chromosomes consist of hundreds to thousands of tandem repeats of TTAGGG fragments [1]. It has been suggested that their function is chromosome end protection and prevention of chromosome fusion [2,3]. Analysis of terminal restriction DNA fragments has shown that the chromosomes of somatic cells lose from 50 to 200 nucleotides of TTAGGG sequence per cell division [4,5]. These changes were explained on the basis of the inability of DNA polymerase to replicate the ends of linear DNA [6]. It was proposed that short telomeres might induce cell senescence [7].

In contrast to somatic cells with a limited life-span, most immortal ones (tumour, germ-line, and stem cells) contain a special enzyme: telomerase [8,9]. It is a ribonucleoprotein complex that synthesizes TTAGGG sequences onto chromosomal ends lengthening the telomeres. The enzyme uses its own RNA template [10] and hence is a kind of reverse transcriptase.

A reverse transcriptase inhibitor, 3′-azido-3′-deoxythymidine (AZT), was shown to suppress the activity of *Tetrahymena* telomerase [11]. We showed that AZT triphosphate inhibited the telomerase activity in extracts of Swiss 3T3 mouse cells [12] and suggested that reverse transcriptase inhibitors (RTI) could block telomerase function in mammalian cells. To check this possibility, we studied RTI effects on telomerase-containing mouse cells in long-term experiments, attempting to produce an artificial senescence process.

NIH and Swiss 3T3 cells and pSV3neo transformed MEF (mouse embryonic fibroblasts) as well as spontaneously transformed MEF cells were used. The latter cells were chosen to reduce the duration of experiments with artificial senescence, since they should have minimal telomere length after the crisis at the beginning of clone formation [3].

2. Materials and methods

2.1. Cells

Primary culture of mouse embryonic fibroblasts (MEF) was initiated from 17–19-day-old CBA mouse fetuses as described in [13]. 3T3 Swiss cells were obtained from Dr. N.F. Sullivan (ICRF, London), pSV3neo transformed MEF and human diploid fibroblasts (HDF) from Dr. A.A. Alimov (EIMB, Moscow), and 3T3 NIH cells from ATCC (USA). The cells were cultivated in DMEM (Gibco) supplemented with 10% fetal bovine serum (Seromed) and 40 μg/ml of gentamycin. The cultures were grown at 37°C in tissue culture dishes (Nunc) under an atmosphere containing 5% CO_2. The cells were replated using trypsin-EDTA when they achieved monolayer status.

Microphotography was performed through a Diaphot (Nikon). DNA synthesis was investigated as described previously [13].

2.2. Spontaneous transformation

After 9–10 population doublings (PD), MEF were placed in 30 mm Petri dishes. Some of the dishes contained media with 3 μM 3′-azido-3′deoxythymidine (AZT) [14], while others contained 20 μM carbovir, a gift from Dr. S.M. Roberts (UK). After decrease in proliferation the media were changed weekly. After 8–12 weeks we observed clone formation.

2.3. Proliferation rate measurement

For growth rate determination we counted the cells for 2 days in succession using the Diaphot microscope with phase-contrast optics. The cells were plated in Petri dishes at a low density (10^3 cells per 30 mm dish) to avoid high-density inhibition. The growth rate was determined as the ratio of the cell number in 50 viewfields to that on the previous day. Growth rate of control cells was taken as 100%.

2.4. Telomerase activity determination

Cell extracts were prepared in accordance with [15]. Extract (10 μl) was mixed with reaction mixture (10 μl) containing 100 mM Tris (pH 8.2), 10 mM dATP, 10 mM dGTP, 0.3 μM [α-^{32}P]TTP (3000 Ci/mmol, 1 mCi/ml), 2 mM $MgCl_2$, 1 mM DTT, and 0.2 μM 5′-d(TTAGGGTTAGGG)-3′ primer. Incubation was performed at 30°C for 1 h. When the reaction had reached completion the mixture was transferred to DEAE paper (10×10 mm), washed with 5% disodium hydrogen phosphate and dried.

Radioactivity was determined in a scintillation counter. For RNA-independent activity determination, DTT was added up to 5 mM, and extracts were treated with RNase A for 15 min at 37°C, 0.03 mg/ml. The results were standardized to the protein content as measured according to Bradford [16]. For a detailed description of the procedure see [12].

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Abbreviations: RTI, reverse transcriptases inhibitor; MEF, mouse embryonic fibroblasts; AZT, 3′-azido-3′-deoxythymidine; ddC, 2′,3′-dideoxycytidine; TAC, telomerase activity

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3. Results and discussion

3.1. Optimal RTI concentrations

To determine the maximal RTI concentrations suitable for long-term experiments, pSV3neo transformed MEF with a high capacity to proliferate were taken as test cells. We found that 3 μM AZT and either 20 μM carbovir or 100 μM dideoxycytidine (Aldrich) (ddC) induced only low growth inhibition (10–20%) in the long-experiments (up to 3 weeks; longer times were not studied). In all further experiments the above-mentioned RTI concentrations were used.

3.2. Obtaining of spontaneous cell transformants

It took 3 months to obtain such clones in the presence of AZT (clone 3) or carbovir (clone 4) and 2 months for control clones, i.e. those formed in the absence of RTI (clones 1,2). The control clones contained TAC, while those obtained in the presence of RTI were TAC-free (Table 1, column A).

This finding requires particular discussion. As could be expected, the long-term experiments revealed that the TAC-free clones had restricted life-spans (25–30 PD). Finally, they lost the capacity to proliferate in the presence of RTI and resembled senescent cells, but resumed proliferation and acquired TAC after removal of RTI (Table 1, column D). It may be suggested that some relatively rare alterations occurred during the formation of TAC-free clones, which reduced the telomere shortening effect on growth inhibition. The life-span extension of the TAC-free clones up to 25–30 PD resembles the lengthening of the life-span of human diploid fibroblasts after transformation with viral nuclear oncoproteins [17]. At any rate, the formation of TAC-free clones in the presence of RTI and the inability of these clones to acquire TAC in the presence of RTI support the assumption that telomerase function is inhibited by AZT and carbovir.

3.3. RTI influence on telomerase activity

To exclude the possibility that RTI can inhibit TAC, we investigated the influence of AZT and carbovir on TAC during continuous cultivation of cells in their presence. Repeated measurements did not reveal any significant changes in TAC after 3 days (Table 1, column B) and after 3 weeks of cultivation (Table 1, column C). Clones 1–3 grew in the presence of AZT, and clone 4 in the presence of carbovir.

3.4. Carbovir-resistant cells

Long-term cultivation (5 months) of clone 4 cells in the presence of carbovir (0, 15, 20 μM) resulted in the formation of cells resistant to carbovir. Their growth rate was independent of carbovir (20 μM). These cells had a very high level of TAC (Table 1, column E) that can be regarded as a suitable explanation for the resistance to carbovir.

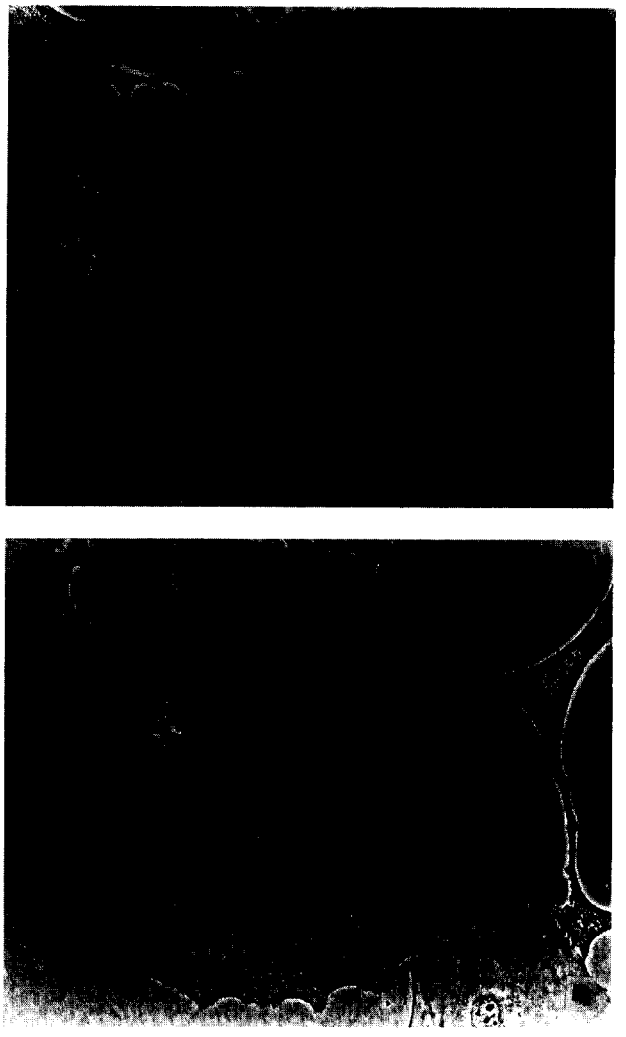

Fig. 1. (A) Senescent MEF and (B) 3T3 Swiss cells, cultivated in the presence of carbovir. Phase contrast, magnification ×160.

The probability of the accidental appearance of two TAC-free clones out of four obtained is 1/16. If the probability of an accidental increase in TAC in carbovir-resistant cells is 50% (in fact, this probability is considerably lower) we can conclude, with a probability of at least 96.875%, that RTI block the telomerase function.

3.5. Induction of artificial senescence

For these experiments we employed clone 2 cells (sponta-

Table 1
Telomerase activity (TAC) of spontaneous transformants

Cells	A	B	C	D	E
Clone 1	7500	6500	7000	–	–
Clone 2	2500	2800	2300	–	–
Clone 3	200	100	200	3200	–
Clone 4	300	200	300	2800	12000
MEF	300	300	0	100	–
HDF	–	100	100	200	200
3T3 Swiss	4000	3200	3500	4100	4000

TAC is measured in arbitrary units [12]. TAC levels up to 500 U did not differ significantly from the background. MEF, HDF and 3T3 Swiss cells were used as negative and positive controls.

Fig. 2. 3T3 Swiss cells, cultivated in the presence of ddC. Phase contrast, magnification ×160.

Fig. 3. Giant mitotic cell. Culture of clone 3 cells after the spontaneous acquisition of TAC and subsequent long-term cultivation in the presence of carbovir. 8 days after removal of carbovir. Phase contrast, magnification ×160.

neously transformed cells with minimal TAC) and both Swiss and NIH 3T3 cells (Table 2).

As demonstrated in Table 2, with the exception of ddC, the sequence of events was similar: after a while proliferation began to decrease gradually; large, senescent-like cells appeared (Fig. 1), the number of these cells increasing. Finally, growth stopped and the cells survived for a long period (more than 4 weeks). The morphology of the large cells resembled that of the senescent ones, described elsewhere [18].

The action of ddC was different and depended on the cell type. There was no effect of 100 μM ddC on 3T3 NIH cells for at least 15 weeks.

At the same time, the proliferation of 3T3 Swiss ceased within 2 weeks. All these cells appeared degenerative: they had massive perinuclear inclusions (Fig. 2) and died over several days. Thus, the effect of ddC on 3T3 Swiss cells resembled the toxic one and differed sharply from that of AZT and carbovir.

The different effects of AZT and carbovir compared with that of ddC may be caused by the structure of the sequence synthesized by telomerase. The TTAGGG repeat does not contain deoxycytidine, therefore its analog cannot influence the function of telomerase.

3.5. Polyploid mitotic cells

The removal of RTI at the stage of the proliferation decrease and the appearance of large cells led to growth restoration. 2 days after the removal of RTI DNA synthesis resumed and then mitotic cells appeared. 7–18 days following the removal of RTI giant mitotic cells were observed (Fig. 3). Standard karyotyping revealed that these mitotic cells were polyploid and some contained more than 600 chromosomes.

We showed previously that TAC was independent of the cell cycle in mouse fibroblasts and the latter possessed TAC at least during a few days of non-proliferation [12]. It may be thus assumed that the removal of RTI restores the telomerase function, leading to telomere elongation, and subsequently to the resumption of cell division.

The above-described induction of senescence-like processes and the appearance of polyploid mitotic cells caused by RTI action, as well as telomerase inhibition by AZT triphosphate in cellular extracts [12], correspond to the finding that RTI blocks the telomerase function. Experiments on the direct measurement of telomere length in RTI treated cells are now in progress in our group. Several attempts at the application of AZT for cancer chemotherapy have recently been described [19]. Our data suggest that among the possible mechanisms of the antitumor action of AZT, its inhibitory effect upon cancer cell telomerase should be considered.

Table 2
Influence of RTI on mouse culture cells

Cells	RTI	Appearance of large cells (weeks)[a]	Cessation of growth (weeks)[b]	Non-dividing cells survival (weeks)
Clone 2	AZT	13	17	>4
Swiss 3T3	AZT	3–6	10–12	>4
	carbovir	3–4	9–11	>4
	mix[c]	2	7	>4
	ddC	2	3	1
NIH 3T3	AZT	5–7	12–16	>4
	carbovir	6–8	16–18	>4
	mix[c]	2–3	9–10	>4
	ddC	no effect	no effect	no effect

[a]Time at which the proportion of large, senescent-like cells (resembled types 5 and 6 according to Bayreuter et al. [18]) exceeded 5%.
[b]Time at which the growth rate fell below one population doubling per 2 weeks.
[c]Mixture of 3 μM AZT and 20 μM carbovir.

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