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Identification of the Tumor Factor of Abnormal Cancer Methylation Enzymes as the Catalytic Subunit of Telomerase

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OBJECTIVE The objective was to study the relationship between the tumor factor of cancer MAT^{LT} and the catalytic subunit of telomerase. The function of telomerase in the blockade of cell differentiation and in the protection of DNA MT resembles closely the function of the tumor factor of cancer MAT^{LT}. Because of this close similarity we made an attempt to examine the possibility that the tumor factor of MAT^{LT} might be the catalytic subunit of telomerase.

METHODS We used purified MAT isozymes, telomerase antibody, immunoprecipitation, and a selective inhibitor of the tumor factor of MAT^{LT} from urine to study the relationship between the tumor factor of MAT^{LT} and telomerase.

RESULTS We were able to show that the tumor MAT^{LT}, but not the liver MAT^{L}, was selectively inhibited by the telomerase antibody, and the tumor MAT^{LT}, but not the liver MAT^{L}, was preferentially immunoprecipitated with the telomerase antibody. The catalytic subunit of telomerase was detectable in the tumor MAT^{LT} preparation by immunoblotting, but was undetectable in the liver MAT^{L} preparation and the tumor MAT^{L} preparation stripped off of the tumor factor. In addition, PP-0.39, which is an effective differentiation inducer purified from urine previously found to selectively antagonize the tumor factor of MAT^{LT}, was found in this study to be a potent inhibitor of telomerase. The inhibition of telomerase by PP-0.39 was far more sensitive than the elimination of the tumor factor from MAT^{LT}.

CONCLUSION All results are consistent with the hypothesis that the tumor factor of MAT^{LT} is the catalytic subunit of telomerase. Thus, the blockade of cell differentiation by telomerase is mediated through its interaction with MAT to affect methylation enzymes, so that hypomethylation of nucleic acids necessary for the cell to undergo differentiation cannot take place.

KEY WORDS: telomerase, tumor factor of MAT^{LT}, methylation enzymes, differentiation.

ABBREVIATIONS
AdoHcy: S-adenosylhomocysteine; Ado-Met: S-adenosylmethionine; Hsp: heat shock protein; MAT: methionine adenosyltransferase; MAT^{L}: the low K_m isozyme of MAT; MAT^{LT}: the tumor factor associated low K_m isozyme of MAT; M_r: molecular weight; MT: methyltransferase; PMSF: phenylmethylsulfonyl fluoride; PP-0.39: pigment peptide with a K_{av} value of 0.39 on the Bio-Gel P2 column; SAHH: S-adenosylhomocysteine hydrolase; TERT: telomerase reverse transcriptase.

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Introduction

Biological methylation is mediated by ternary methylation enzymes consisting of MAT-MT-SAHH[1]. These enzymes play an important role in the regulation of cell replication and differentiation because of their critical roles in the regulation of ribosome production[2], which in turn dictates the commitment of the cell to enter cell cycle[3], and the expression of tissue specific genes[4]. Methylation enzymes are under the stringent control of growth factors. As a matter of fact, SAHH is a steroid hormone receptor[1]. The growth factor such as a steroid hormone promotes methylation enzymes to the stable and functional ternary enzymes for efficient methylation of nucleic acids to facilitate cell replication. In the absence of growth factor, ternary methylation enzymes dissociate into individual enzymes which are quickly degraded to cause hypomethylation of nucleic acids, which is a critical process for the cell to undergo terminal differentiation[5]. Differentiation requires hypomethylation of pre-rRNA to decelerate ribosome production to put a halt on cell cycle progression and hypomethylation of the promoters of tissue specific genes repressed by DNA methylation. Both methylations of pre-rRNA and DNA take place during the very brief moment of nascent stage right after synthesis[6-8]. The efficiency of methylation to keep up with polymerase activity is an important factor that determines whether the cell will continue cell cycle replicating or undergo differentiation. We have previously observed that methylaton enzymes are much more stable in the ternary enzyme complex, and the efficiency of methylation is better with AdoMet generated by MAT than that provided exogenously[1,9]. Therefore, factors that are capable of influencing the integrity of methylation enzymes are imminently important for the regulation of cell replication and differentiation.

Besides growth factors to exercise positive influence on the integrity of ternary methylation enzymes, we have discovered a tumor factor which also had a great influence on methylation enzymes. This tumor factor was associated with MAT in cancer cells[9-11]. This association changed the kinetic property of MAT. The tumor isozyme MAT^{LT} displayed a K_m value 7-fold greater than the normal isozyme MAT^{L}, which had a K_m value of 3 μM methionine. MAT^{L} is the isozyme involved in the methylation of nucleic acids. The increased K_m value of the tumor MAT^{LT} implies that cancer methylation enzymes retain more AdoMet, and the higher level of enzyme bound AdoMet offers a greater stability to cancer methylation enzymes. Ternary tRNA methylation enzymes of cancer cells could survive pH 5 precipitation and redissolution by raising pH back to 7.4, whereas the same treatment caused the normal tRNA methylation enzymes to dissociate into individual enzymes[9]. Prudova et al.[12] observed that the binding of AdoMet stabilized cystathionine β-synthase against proteolytic degradation by 12 Kcal/mol. Therefore, the association of the tumor factor with MAT^{LT} is a contributing factor to stabilize the integrity of ternary methylation enzymes. The tumor factor by virtue of increasing the K_m value of MAT locks methylation enzymes in an exceedingly stable and active state to ensure efficient methylation of nucleic acids, so that the hypomethylation of nucleic acids which is necessary for the cell to undergo differentiation cannot take place. Elimination of the tumor factor of abnormal cancer methylation enzymes is therefore an attractive strategy to induce terminal differentiation of cancer cells[13,14]. The finding of Chiba et al.[15] in essence supports our argument that the tumor factor of abnormal methylation enzymes is responsible for the blockade of differentiation. They found that the pool sizes of both AdoMet and AdoHcy shrank greatly when cancer cells were induced to undergo terminal differentiation. CDA-2, a preparation purified from fresh human urine, provides an effective solution to achieve induction of differentiation of cancer cells by selectively antagonizing the tumor factor of MAT^{LT}[14]. CDA-2 was approved by the state FDA of China in 2004 for cancer therapy[16].

Telomerase has also been widely implicated to be involved in the regulation of cell differentiation with the obvious reason that telomerase is down-regulated upon induction of differentiation of immortal cells and cancer cells[17-21]. The down-regulation of telomerase appears to precede differentiation, suggesting that telomerase plays an active role to blocking differentiation[22]. The mechanism allowing telomerase to block cell differentiation, however, remains unknown. It is obviously unrelated to the maintenance of telomere, which is the primary function of telomerase to immortalize cell replication. It was revealed that telomerase is capable of protecting DNA MT[23]. The function of telomerase in the blockade of cell differentiation and the protection of DNA MT closely resembles the function of the tumor factor of MAT^{LT} described above. Because of this close similarity, we made an attempt to examine the possibility that the tumor factor of MAT^{LT} might be the catalytic subunit of telomerase. This was not a far-fetched hypothesis, since telomerase was found to become associated with many cellular proteins such as Hsp90[24,25], NF-KB[26], KIP[27], poly(ADP-ribose) polymerase[28], pinX1[29] and nucleolin[30]. Besides, we have previously found that CDA-2 is capable of inhibiting telomerase in addition to antagonizing the tumor factor of MAT^{LT}[31].

Materials and Methods

Propagation of rat Novikoff ascites hepatoma cells

Rat Novikoff hepatoma cells were purchased from

ATCC, which was initially grown as suspension culture in ISCOVE'S modified Dulbecco's medium (Sigma, St. Louise, MO, USA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and penicillin (50 units/ml)-streptomycin (50 µg/ml). Cells were harvested by centrifugation, washed and suspended in PBS to make 10^7 cells/ml. Each Sprague Dawley rat (female, 150-175 g, from Timco Breeding Laboratories in Houston, TX, USA) was inoculated 1 ml of cell suspension into the abdominal cavity. Thereafter, ascites fluid collected from the rat abdomen one week after transplantation was used for serial transplantation, inoculating 1 ml ascites fluid per rat.

Culture of HL-60 cells

HL-60 cells were purchased from ATCC, and were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum, penicillin (50 units/ml) and streptomycin (50 µg/ml). Cells were subcultured every 3 to 4 days at an initial concentration of 10^5 cells /ml.

Purification of MAT

Novikoff ascites hepatoma cells were sedimented from ascites fluid at 2,000 × g for 10 min, and washed once with 4 volumes of 0.05 M Tris-HCl (pH 7.4), 0.25 M sucrose, 5 mM $MgCl_2$, and 1 mM PMSF. The washed cells were suspended in 4 volumes of the above solution, and passed through a chilled French pressure cell at 3000-5000 psi to disrupt cell and nuclear membranes to release soluble materials. The suspension was centrifuged at 5,000 × g for 20 min, and then 48,400 × g for 2 h to obtain cytosol. The cytosol was adjusted to pH 5 with 0.1 N acetic acid. The precipitate was collected by centrifugation at 5,000 × g for 10 min, and washed once with 5 volumes of 0.05 M Tris-HoAc (pH 5), 5 mM $MgCl_2$, and 0.1 mM PMSF. The washed sediment was redissolved in 5 volumes of 0.05 M Tris-HCl (pH 7.4), 5 mM $MgCl_2$, and 0.1 mM PMSF, pH readjusted to 7.4. Insoluble materials were removed by centrifugation at 5,000 × g for 10 min. The supernatant (7.5 ml from 7.5 ml packed cells) was passed through a column of DEAE-sephadex, 1.7 cm × 15 cm, for the purification of MAT as previously described[9]. MAT purified in this way from Novikoff ascites hepatoma cells was termed MAT^{LT}, which displays a K_m value of 23 µM methionine[9].

MAT^L was purified from pH 5 precipitable tRNA methylation enzymes of rat liver as above described except that Potter homogenizer was used to disrupt cell membrane. Rat livers were minced through a Harvard tissue press to remove connective tissue. The minced liver was homogenized with 4 volumes of 0.05 M Tris-HCl (pH 7.4), 0.25 M sucrose, 5 mM $MgCl_2$, and 1 mM PMSF. The purification of MAT from the pH 5 precipitate was as above described. MAT so purified from rat liver was termed MAT^L, which displayed a K_m value of 3 µM methionine[9].

Conversion of the tumor MAT^{LT} into the normal isozyme MAT^L

One third of the pooled 24 ml of the active tumor MAT^{LT} fractions from DEAE-sephadex chromatography above described was dialyzed against 500 ml of 0.05 M Tris-HCl (pH 8.4), 0.5 mM EDTA, 5 mM $HSCH_2CH_2OH$, and 0.1 mM PMSF at 4°C for 48 h. The dialysate was passed through a column of DEAE-sephadex, 0.9 cm × 15 cm, preequilibrated with the dialysis buffer. The column was eluted with a 100 ml linear gradient of KCl from 0.05 to 0.5 M in the dialysis buffer as above described. MAT so obtained displayed a K_m value of 3 µM similar to that of the liver MAT^L as previously described[9]. Under these conditions, the tumor factor of MAT^{LT} became dissociated from the enzyme, and was eluted in the fractions before the active enzyme peak[9]. The pooled enzyme fractions and the inactive proteins before the enzyme peak were separately dialyzed against 0.05 M Tris-HCl (pH 7.4), 5 mM $MgCl_2$, and 0.1 mM PMSF at 4°C for 48 h.

Two thirds of the pooled 24 ml of the active tumor MAT^{LT} fractions was dialyzed against 1 liter of 0.05 M Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.1 mM PMSF at 4°C for 48 h. One half of this dialysate was passed through a column of DEAE-sephadex, 0.9 cm × 15 cm, preequilibrated with the dialysis buffer. The column was eluted with a 100 ml linear gradient of KCl from 0.05 to 0.5 M in the dialysis buffer as above described. The enzyme purified in this way retained the tumor factor to display a K_m value of 23 µM methionine[9].

Immunoprecipitation, SDS-PAGE gel electrophoresis, and immunoblotting

Dialyzed MAT preparations and inactive protein preparation containing approximately 0.2 mg protein in 0.05 M Tris-HCl (pH 7.4), 5 mM $MgCl_2$, and 0.1 mM PMSF were incubated with 20 µg of rabbit polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) raised against amino acids 900-1130 mapping at the C terminus of TERT for 1 h in the ice bath. One hundred µl of protein G PLUS-agarose (Santa Cruz Biotechnology) was added, and the mixture was stirred with a magnetic stirrer for 1 h in the cold room of 4°C. Agarose was sedimented by centrifugation at 5,000 × g for 5 min at 4°C, and washed three times with 20 mM HEPES (pH 7.6), 20% glycerol, 100 mM NaCl, 0.2 mM EGTA, 1 mM $MgCl_2$, 0.1% Nonidet P-40, and 0.1% bovine serum albumin. The washed agarose pellet was suspended in 50 µl of the wash buffer and heated to 80°C for 10 min to extract protein. Agarose was removed by centrifugation at 5,000 × g for 5 min. The supernatant was mixed with one third volume of 0.25 M Tris-HCl (pH 6.8), 8% SDS, 35% glycerol, 2.5% $HSCH_2CH_2OH$, and 5 mg/ml bromophenol blue. Fifteen µl of this solution was taken for SDS-PAGE electrophoresis.

SDS-PAGE electrophoresis was carried out on a gel made up by 10% separating gel, 5 mm in thickness and

6 cm in length, and 4% stacking gel, 2 cm in length according to the standard procedure. Electrophoresis was carried out in the cold room of 4°C at 60 V for 17 h with the electrophoresis buffer 25 mM Tris-HCl (pH 8.3), 0.192 M glycine, and 0.1% SDS. After electrophoresis, the proteins on the gel were transferred to the nitrocellulose membrane by electrophoresis at 40 V for 3 h with the transfer buffer 25 mM Tris-HCl (pH 8.3), 0.192 M glycine, 0.01% SDS, and 20% methanol. The first left lane of the membrane with M_r markers was cut off to be stained with 0.1% brilliant blue. The membrane containing samples of immunoprecipitates was stained with IgG conjugated alkaline phosphatase staining solution (Santa Cruz Biotechnology) according to the instruction provided. After a blocking step using 5% fat free dry milk dissolved in TBST [0.01 M Tris-HCl (pH 7.5), 0.15 M NaCl, and 0.2% Tween 20] overnight at 4° C with constant agitation, the membrane was incubated with the primary anti-telomerase antibody (rabbit polyclonal IgG by Santa Cruz Biotechnology), 2 μg/ml in TBST for 1.5 h at room temperature. The membrane was then washed three times with TBST, each time for 5 min, followed by incubation with the anti-rabbit IgG alkaline phosphatase-conjugated secondary antibody reagent diluted by TBST 1:10,000 for 1 h at room temperature with agitation. The membrane was again washed 5 times with TBST, each time for 5 min, followed by incubation with western blue stabilized substrate for alkaline phosphatase until the bands have reached the desired intensity within 8 min. The membrane was finally washed with TBST, and the photograph was taken for the record.

Purification of differentiation inducer PP-0.39 (PP stands for pigment peptide) from human urine

Freshly collected human urine was adjusted to pH 8.2, and the resulting white flocculent precipitate was removed by filtration. After dilution with 2 volumes of purified Milli-Q water, 24 liters of the diluted urine were passed through 100 ml of DEAE- sephadex packed on a Millipore filtration apparatus. DEAE-sephadex was successively eluted stepwise with 200 ml each of 0.15 M, 0.4 M, and 0.8 M NaCl. PP-0.39 was present in the 0.8 M NaCl eluant, which became dark brown flocculent precipitate after adjusting pH to 1.5 with 2 N HCl. The precipitate was collected by centrifugation at 5,000 × g for 10 min. The precipitate obtained from 80 liters of urine was suspended in 8 ml of 25 mM phosphate buffer, pH 7.8 and 1 N NaOH was added dropwise to dissolve the precipitate. pH was maintained below 8. Insoluble materials were removed by centrifugation at 5,000 × g for 10 min. The supernatant from 40 liters of urine about 5 ml was applied to a column of Bio-Gel P2, 2.5 cm × 88 cm, for the purification of differentiation inducers by gel filtration. The elution was carried out with 25 mM phosphate buffer, pH 7.8 at a flow rate of approximately 4 ml/tube/6 min. An aliquot of 50 μl was withdrawn from each tube to dilute with 2 ml of water for the determination of absorption at A_{280}. PP-0.39 was present in the peak with a K_{av} value of 0.39, which was recovered by precipitation at pH 1.5 and redissolution in NaOH as above described. The yield was 27 mg from 80 liters of urine.

Assay procedures

Telomerase activity was measured by the $TRAP_{EZE}$ RT Telomerase Detection Kit (Chemicon, MA, USA), which is a highly sensitive in vitro assay for the detection and real time quantification of telomerase activity. Telomerase was prepared from HL-60 cells by incubations of 10^6 cells in 200 μl CHAPS lysis buffer in an ice bath for 30 min. Enzyme extract was obtained by centrifugation at 12,000 × g for 20 min at 4°C. Real-time PCR was performed for one cycle of 30°C for 30 min and 95°C for 2 min, followed by 45 cycles of 15 sec at 94°C, 60 s at 59°C and 10 s at 45°C employing 7500/7500 Fast Real Time PCR System (Applied Biosystems, CA, USA).

MAT was assayed as described previously[9] except that the water miscible counting fluor was made by dissolving 4 g of Omnifluor (Perkin Elmer Life and Analytical Sciences, Boston, MA, USA) in one liter of toluene-methoxyethanol (6:4). NBT assay was conducted as previously described[13]. Protein was determined by the procedure of Lowry et al.[32]

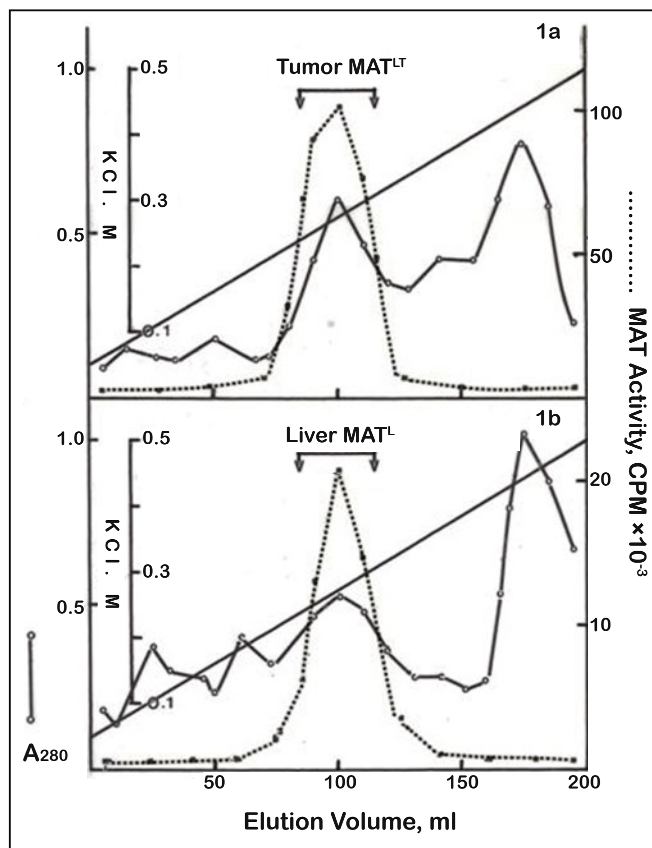
Results

Purification of MAT

MAT was purified from ternary tRNA methylation enzymes precipitable at pH 5 from cytosol to avoid possible contamination of Hsp90 which was known to associate with telomerase in cancer cells[24,25]. As previously described[9], pH 5 treatment brought down 28.7% of the MAT^{LT} activity presented in the cytosol of Novikoff hepatoma cells, but only 10.6% of the MAT^{L} activity of the liver cytosol. Purification of pH 5 precipitable MAT was carried out as described in the materials and methods section. The results are presented in Fig.1. The enzyme peak is located in the A_{280} absorption peak eluted between 0.24 and 0.3 M KCl.

Conversion of the tumor MAT^{LT} into the normal isozyme MAT^{L}

We have previously found that when the tumor MAT^{LT} was exposed to combined effects of high salt, EDTA, $HSCH_2CH_2OH$, and higher pH, the tumor factor of MAT^{LT} became dissociated from the enzyme[2]. One third of the pooled 24 ml tumor MAT^{LT} of Fig.1a was dialyzed against 500 ml 0.05 M Tris-HCl (pH 8.4), 0.5 mM EDTA, 5 mM $HSCH_2CH_2OH$, and 0.l mM PMSF at 4°C for 48 H. The dialysate was rechromatographed on a column of DEAE-sephadex, 0.9 cm × 15 cm, preequilibrated with the dialysis buffer as described in materials and methods. The pooled inactive proteins eluted be-



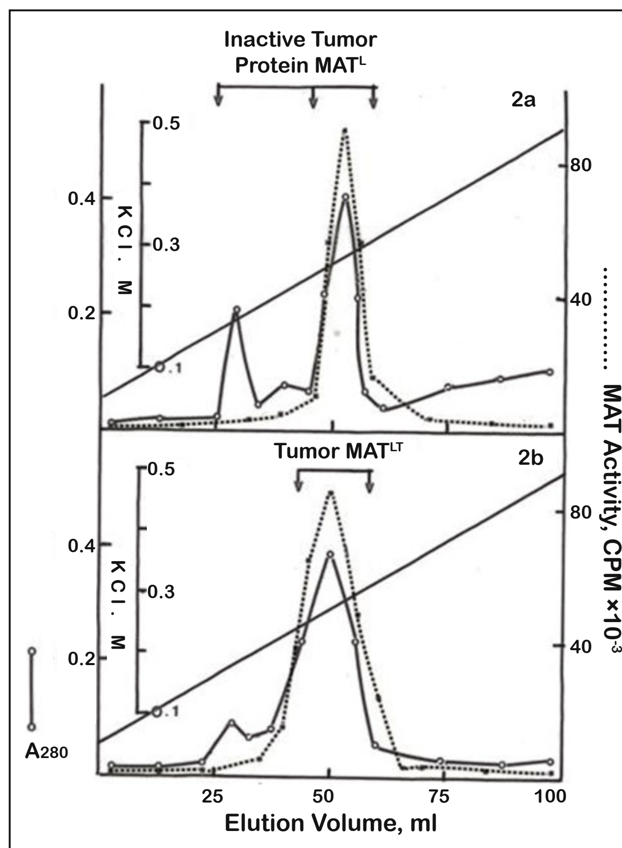


Fig.l. Purification of MAT^{LT} from Novikoff ascites hepatoma cells and MAT^{L} from rat liver. pH 5 precipitate, approximately 1.5 ml each, of the cytosol prepared from 7.5 ml Novikoff ascites hepatoma cells (1a) or 16.2 g rat liver (1b) was redissolved in 5 volumes of 0.05 M Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.1 mM PMSF, and pH readjusted to 7.4. Insoluble materials were removed by centrifugation at 5,000 × g for 10 min. Each supernatant was passed through a column of DEAE-sephadex, 1.7 cm × 15 cm, for the purification of MAT as described in materials and methods. The A_{280} profile was determined by a manual reading of A280 from each tube, and the MAT profile was determined by withdrawing an aliquot of 35 µl from each tube to react with 15 µl reaction mixture containing 0.5 µCi of [3H-CH3]methionine (70 Ci/mmole from Perkin Elmer Life Sciences, Waltham, MA, USA) for the assay of the tumor MATLT, or 1 µCi of [3H-CH3]methionine for the assay of the liver MATL.

Fig.2. Conversion of the tumor MAT^{LT} into the normal MAT^{L}. One third of the pooled 24 ml of the tumor MAT^{LT} of Fig.1a was dialyzed against 500 ml of 0.05 M Tirs-HCl (pH 8.4), 0.5 mM EDTA, 5 mM $HSCH_2CH_2OH$, and 0.1 mM PMSF at 4°C for 48 h. The dialysate (2a) was rechromatographed on a column of DEAE-sephadex, 0.9 cm × 15 cm, preequilibrated with the dialysis buffer as described in materials and methods. Two thirds of the pooled 24 ml of the tumor MAT^{LT} was dialyzed against 1 liter of 0.05 M Tris-HCl (pH 7.4), 5 mM $MgCl_2$, and 0.1 mM PMSF at 4°C for 48 h. One half of this dialysate (2b) was rechromatographed on a column of DEAE-sephadex, 0.9 cm × 15 cm, preequilibrated with the dialysis buffer as described above. The A_{280} profile and the MAT profile were determined as described in Fig.1.

tween 0.15 and 0.24 M KCl, and the tumor MAT^{L} eluted between 0.24 and 0.3 M KCl as shown in Fig.2a were separately dialyzed against 500 ml of 0.05 M Tris-HCl (pH 7.4), 5 mM $MgCl_2$, and 0.1 mM PMSF. The tumor MAT^{L} so obtained displayed a K_m value of 3 µM methionine similar to that of the liver MAT^{L}[9].

Two thirds of the pooled 24 ml tumor MAT^{LT} of Fig. 1a was dialyzed against 1 liter 0.05 M Tris-HCl (pH 7.4), 5 mM $MgCl_2$, and 0.1 mM PMSF at 4°C for 48 h. One half of this dialysate was rechromatographed on a column of DEAE-sephadex, 0.9 cm × 15 cm, preequilibrated with the dialysis buffer as described in materials and methods. The enzyme so purified as shown in Fig.2b still retained the tumor factor to display a K_m value of 23 µM methionine[9]. The pooled enzyme was dialyzed against 500 ml of 0.05 M Tris-HCl (pH 7.4), 5 mM $MgCl_2$, and 0.1 mM PMSF at 4°C for 48 h.

Selective inhibition of the tumor MAT^{LT} by the telomerase antibody

The telomerase antibody selectively inhibited the activity of tumor MATLT, while affected very little the activity of liver MATL as shown in Table 1. The degree of inhibition depended on the ratio of antibody/enzyme, the higher the ratio of antibody/enzyme the greater the inhibition. These results strongly suggest that there may exist a physical linkage between the tumor MAT enzyme and telomerase. Therefore, the interaction between the antibody and telomerase ultimately affects the activity of MAT. There is no such effect demonstrable in the liver MATL preparation.

Table 1. Selective inhibition of the tumor MATLT by the telomerase antibody.

Antibody used, µg	Tumor MAT^{LT} dilution			Liver MAT^{L} dilution	
	No	1:5	1:10	No	1:5
0	121,567 (100%)	32,100 (100%)	13,104 (100%)	94,501 (100%)	16,064 (100%)
0.125	97,057 (90%)	25,620 (80%)	6,546 (49%)	96,305 (102%)	15,261 (95%)
0.25	91,326 (75%)	17,465 (54%)	3,423 (25%)	90,456 (99%)	14,614 (91%)
0.375	70,656 (58%)	12,186 (38%)	1,677 (13%)	90,720 (96%)	13,494 (86%)

Twenty five µl aliquots of the dialyzed enzyme preparations from Fig.1 without dilution, or 1 to 5 dilution, or 1 to 10 dilution with 0.05 M Tris-HCl (pH 7.4), 5 mM $MgCl_2$, and 0.l mM PMSF were incubated with 10 µl of the telomerase antibody preparation containing different amounts of antibody as indicated in the ice bath for 1 h, and then 15 µl of incubation mixture was added to each tube for the assay of enzyme activity. The undiluted tumor MATLT and liver MATL had protein content of 60 µg and 100 µg per ml, respectively. The tumor MATLT was assayed with 1 µCi [3H-CH3]methionine, and the liver MATL was assayed with 5 µCi [3H-CH3]methionine, respectively. Data are presented as CPM (% Activity).

Preferential immunoprecipitation of the tumor MAT^{LT} with the telomerase antibody

In Table 1, we have shown that the tumor MAT^{LT} was selectively inhibited by the telomerase antibody, which was dependent on the amount of antibodies. It may take more than one antibody per molecule of MAT^{LT} to inactivate the enzyme activity. As shown in Table 2, a significant proportion of the tumor MAT^{LT} not inhibited by the telomerase antibody cosedimented with the telomerase antibody, but not much of the liver MAT^{L} was brought down by the telomerase antibody. These results also suggest a linkage between the tumor MAT enzyme and telomerase to cause the cosedimentation.

Table 2. Preferential immunoprecipitation of the tumor MAT^{LT} with the telomerase antibody.

% MAT activity remaining in the protein G PLUS-agarose supernatant		
MAT/Telomerase antibody	Tumor MAT^{LT}	Liver MAT^{L}
12:1	81,693 (84%)	84,336 (105%)
3:1	18,763 (36%)	76,304 (95%)
1:1	319 (1%)	66,665 (83%)

One hundred µl aliquots of the dialyzed MAT preparations of Fig.1 were incubated with the telomerase antibody in the ratios of enzyme protein to telomerase antibody 12:1, 3:1, and 1:1 in the ice bath for 1 h. Twenty five µl aliquots were withdrawn for the assay of MAT activity as described in Table 1. Twenty µl of protein G PLUS-agarose suspension was then added to each tube to incubate at 4o C for 1 h. Tubes were constantly shaken during the incubation with protein G-PLUS-agarose. Protein G-PLUS-agarose was sedimented by centrifugation at 5,000 × g for 5 min. Twenty five µl aliquots were again withdrawn from the supernatant for the assay of MAT activity to determine the percent of activity remaining in the supernatant. Data are presented as CPM (% of the total CPM detectable before centrifugation).

Demonstration of the presence of telomerase in the MAT^{LT} preparations by immunoblotting

Results presented in Table 1 and 2 suggest that there may exist a linkage between telomerase and the tumor MAT. Immunoprecipitation, SDS-PAGE gel electrophoresis, and immunoblotting were carried out to search for the additional evidence of the linkage between telomerase and the tumor MAT^{LT} enzyme. Dialyzed MAT preparations and inactive protein preparation described in Fig.1 and 2 containing approximately 200 µg protein each were incubated with 20 µg of the telomerase antibody in the ice bath for 1 h. Immunoprecipitates were sedimented with protein G PLUS-agarose, extracted, separated by PAGE gel electrophoresis, transferred to the nitrocellulose membrane, and immunoblotted with IgG conjugated alkaline phosphatase as described in materials and methods. Results are presented in Fig.3. Lane 1 is the liver MAT^{L} which does not have telomerase. Lane 2 is the tumor MAT^{LT} of Fig.1a, and Lane 3 is the tumor MAT^{LT} of Fig.2b. Both have two major bands of telomerase. One migrates slightly behind the marker with M_r 116K, which is most likely the intact telomerase with a M_r 120K (Santa Cruz Biotechnology). The other major band migrates slightly behind the intact telomerase band with a M_r approximately 140-150K. This band may represent the telomerase in association with another protein component which may or may not be a part of MAT enzyme. Degraded products which migrate faster than the intact telomerase are also evident. Lane 4 is the inactive proteins of Fig.2a which shows only the degraded product of telomerase migrating almost to the end of the gel. Lane 5 is the tumor MAT^{L} of Fig.2a which does not have telomerase like the liver MAT^{L}. However, the residual band with M_r approximately 140-150 and the degraded products are still detectable. These results clearly show that telomerase is associated with the tumor MAT^{LT}, and once the tumor factor becomes dissociated from the enzyme, telomerase also becomes no longer associated with the enzyme.

Purification of differentiation inducer PP-0.39 from human urine

Differentiation inducer PP-0.39 was purified from freshly collected human urine as described in materials and methods. In our previous study[5], this differentiation inducer was designated as PP-0, because it was eluted in the void fraction with a K_{av}= 0 in the purification step in-

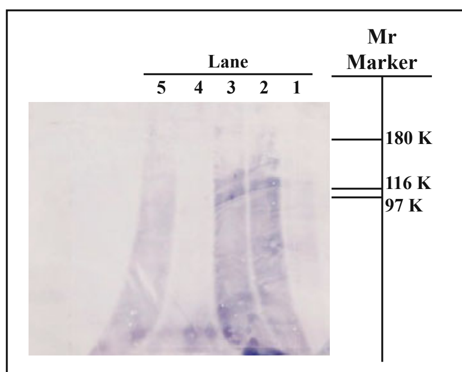


Fig.3. Demonstration of the presence of telomerase in the MAT^{LT} preparations by immunoblotting. Dialyzed MAT preparations and inactive protein preparation described in Fig.1 and 2 containing approximately 200 μg protein each were incubated with 20 μg of the telomerase antibody in the ice bath for 1 h. One hundred μl of protein G PLUS-agarose was added, and the mixture was stirred with a magnetic stirrer for 1 h in the cold room at 4°C. Subsequent sedimentation, extraction, SDS-PAGE gel electrophoresis and immunoblotting were carried out as described in materials and methods. Lane 1 is the liver MAT^{L}. Lane 2 is the tumor MAT^{LT} of Fig. 1a. Lane 3 is the tumor MAT^{LT} of Fig.2b. Lane 4 is the inactive protein of Fig.2a. Lane 5 is the tumor MAT^{L} of Fig.2a.

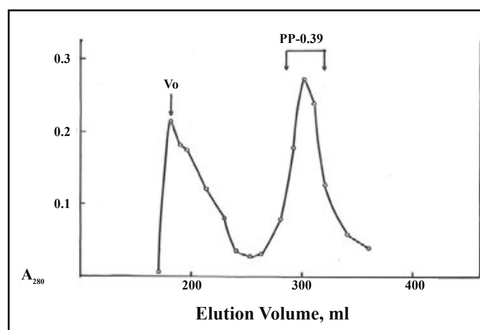


Fig.4. Purification of PP-0.39 by gel filtration on Bio-Gel P2. The acid precipitate obtained from 0.8 M NaCl eluant of DEAE-sephadex adsorbed materials from 80 L of human urine as described in material and methods was redissolved in NaOH as described in materials and methods. Approximately 5 ml of the dissolved supernatant from 40 liters urine was put on Bio-Gel P2 column, 2.5 cm × 88 cm, for the purification of differentiation inducers by gel filtration. The elution was carried out with 25 mM phosphate buffer, pH7.8, collecting approximately 4 ml/tube/6 min. An aliquot of 50 μl was withdrawn from each tube to dilute with 2 ml of water for the determination of A_{280} by manual reading.

volving gel filtration on AcA202 gel column. 0 of PP-0 referred to the K_{av} value of this component. AcA202 was no longer available, so we had to use Bio-Gel P2 as the substitute. In the Bio-Gel P2 column, this component was eluted in the peak with a K_{av} value of 0.39 as shown in Fig.4. This differentiation inducer is now designated as PP-0.39. PP-0.39 and gave rise to characteristic dark brown flocculent precipitate when the solution was acidified. The UV absorption peak at V_o did not produce such characteristic precipitate when acidified. Therefore most of UV absorbing materials of V_o were also excluded from the previous PP-0 preparation, because PP-0 was also recovered by acid precipitation. The inhibitory effect of PP-0.39 against the tumor MAT^{LT} and the spleen MAT^{L} is shown in Fig.5. The tumor MAT^{LT} was inhibited to a greater extent than the spleen MAT^{L}. The spleen MAT^{L} was only significantly inhibited above 0.4 mg/ml. The inhibitory effect against the tumor MAT^{LT} prepared from rat Novikoff hepatoma cells is almost identical as the inhibition of the tumor MAT^{LT} prepared from HL-60 cells by PP-0[5]. In the previous study[5], we have demonstrated that the inhibition of the tumor MAT^{LT} by PP-0 was attributable to the selective antagonistic effect on the tumor factor of MAT^{LT}. It required 0.25 mg/ml of PP-0 to completely eliminate the effect of the tumor factor to convert the tumor MAT^{LT} into the normal isozyme MAT^{L} by the *in vitro* enzyme assay[5]. We would like to know how this differentiation inducer would affect telomerase activity. If the tumor factor of MAT^{LT} is the catalytic subunit of telomerase as the evidence shows, this differentiation inducer should also affect telomerase activity.

Potent inhibition of telomerase activity by PP-0.39

Telomerase activity was assayed based on fluorometric detection by employing the $TRAP_{EZE}$ Telomerase Detection Kit of Chemicon International. To obtain valid quantitative results using the $TRAP_{EZE}$ Telomerase Detection Kit, the production of a standard curve using the TSR8 template and the inclusion of the assay controls were necessary. A typical standard curve so obtained is shown in Fig.6.

Twenty five μl reaction mixture in each well included 5 μl 5 × $TRAP_{EZE}$ Reaction Mix, 0.4 μl Tag Polymerase (5 units/μl), 2 μl enzyme extract, and 17.6 μl of nuclease free water containing various amounts of PP-0.39. The real-time experiment was conducted to include the PCR parameters described in materials and methods to obtain average Ct values. The corresponding amount of TSR8 was derived from the standard curve shown in Fig.6. The data presented in Table 3 clearly show that PP-0.39 is a potent inhibitor of telomerase, reaching above 99% inhibition at 10 μg/ml. At 30 μg/ml, the enzyme activity is totally undetectable. The inhibitory effect of PP-0.39 on telomerase is far more sensitive than its effect on the tumor factor of MAT^{LT}, which required 0.25 mg/ml in the in vitro enzyme assay to eliminate the tumor factor for the conversion of the tumor MAT^{LT} into MAT^{L} [5]. Telomerase activity of HL-60 cells was also effectively inhibited by PP-0.39 in cell culture as shown in Table

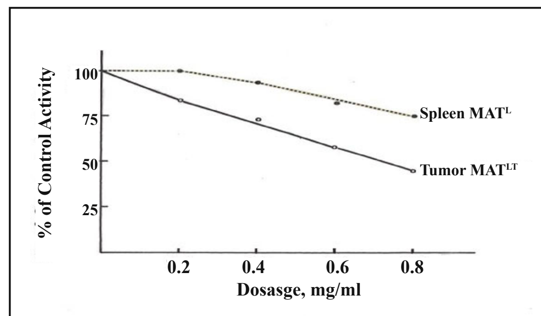


Fig.5. Inhibitory effects of PP-0.39 on the tumor MAT^{LT} and the spleen MAT^{L}. The tumor MAT^{LT} and the spleen MAT^{L} were purified from the cytosol of rat Novikoff hepatoma cells and rat spleen, respectively, by DEAE-sephadex chromatography as described in Fig.1. The inhibitory effects were studied by the addition of 10 µl of PP-0.39 of the indicated dosage to 25 µl of the enzyme preparation, and then 15 µl of reaction mixture containing 0.5 µCi of $[^3H\text{-}CH_3]$methionine were added for the assay of enzyme activity as described in materials and methods.

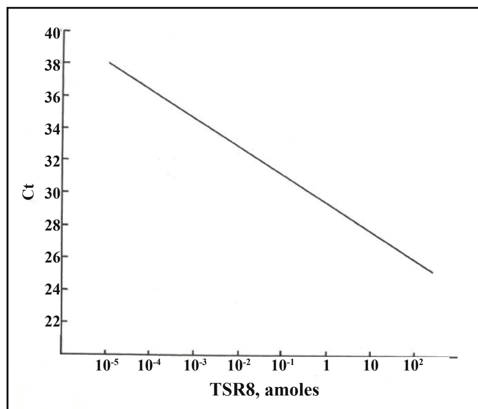


Fig.6. TSR8 Standard Curve. TSR8 is an oligonucleotide with a sequence identical to the TS primer extended with 8 telomeric repeats AG $(GGTTAG)_7$. The stock TSR8 concentration provided within the $TRAP_{EZE}$ RT Telomerase Detection Kit is 20 amoles/µl. Amole is the telomerase activity unit assessed by the amount of extended primers with telomeric repeats. Prepared 1:10 serial dilutions of the stock concentration with CHAPS lysis buffer to obtain TSR concentrations of 2 amoles/µl, 0.2 amoles/µl, and 0.02 amoles/µl. Performed the $TRAP_{EZE}$ RT assay using 2 µl of each TSR8 dilution including the 20 amoles/µl stock concentration. Plot each data point based on the Ct measurements of the reaction that were performed with the TSR8 dilution using real time instrument software, a standard graphing program. Plot the $\log_{10}$ on the X-axis against the average Ct value of the corresponding concentration of TSR8 on the Y-axis.

Table 3. Inhibition of telomerase activity in vitro by PP-0.39.

		PP-0.39			
Well	Control	1 µg/ml	3 µg/ml	10 µg/ml	30 µg/ml
Average Ct	26.40 ± 0.084	27.50 ± 0.035	28.84 ± 0.055	31.22 ± 0.187	36.98 ± 1
TSR8, amoles	54.80 ± 6.03	12.66 ± 0.59	2.15 ± 0.16	0.09 ± 0.022	0
% of Control activity	100	23.10	3.92	0.16	0

Twenty five µl reaction mixture in each well included 5 µl 5 × $TRAP_{EZE}$ RT Reaction Mix, 0.4 µl Taq Polymerase (5 units/µl), 2 µl enzyme extract from HL-60 cells, and 17.6 µl nuclease free water containing various amount of PP-0.39 as indicated. The real-time experiment was conducted to include PCR parameters described in Experimental Procedures to obtain average Ct values. Data are expressed as means±SD, $n = 3$ in each group. The amount of TSR8 was derived from the standard curve shown in Fig.6.

Table 4. Inhibition of telomerase activity of HL-60 cells by PP-0.39 in cell culture.

		PP-0.39		
Well	Control	12 µg/ml	24 µg/ml	36 µg/ml
Average Ct	26.40 ± 0.084	27.17 ± 0.107	30.39 ± 0.154	31.11 ± 0.09
TSR8, amoles	54.80 ± 6.03	1.39 ± 0.201	0.279 ± 0.06	0.107 ± 0.013
% of Control activity	100	2.54	0.51	0.19

HL-60 cells were subcultured at an initial concentration of 10^5 cells/ml with or without the indicated amount of PP-0.39. After incubation for 3 days, 10^6 cells from each flask were sedimented for enzyme extraction as described in materials and methods. Aliquots of 2 µl were withdrawn for the assay of telomerase activity as described in Table 3.

4. By incubation of 3 days with relative low dosages of PP-0.39 between 12 and 36 µg/ml, telomerase activity was inhibited to more than 97%. At such low dosages, the induction of NBT-positive cells only reached modest levels between 15 and 33%. It is obvious, that the inhibition of telomerase activity is far more sensitive than the elimination of the tumor factor from MAT^{LT}. It required 0.3 mg/ml of PP-0 in the previous study[5] to induce 65% of NBT-positive cells and to effectively convert the tumor MAT^{LT} into MAT^{L}.

Discussion

We have previously identified the tumor factor of MAT^{LT} to play an essential role in the blockade of cell differentiation, and in this study we have identified this tumor factor as the catalytic subunit of telomerase. This identification solves the puzzle on the mechanism of telomerase to block cell differentiation. The blockade of differentiation by telomerase is mediated through its interaction with MAT^{L} to increase the K_m value 7-fold, which enables the enzyme to hold more AdoMet. The increased content of AdoMet provides a stabilizing ef-

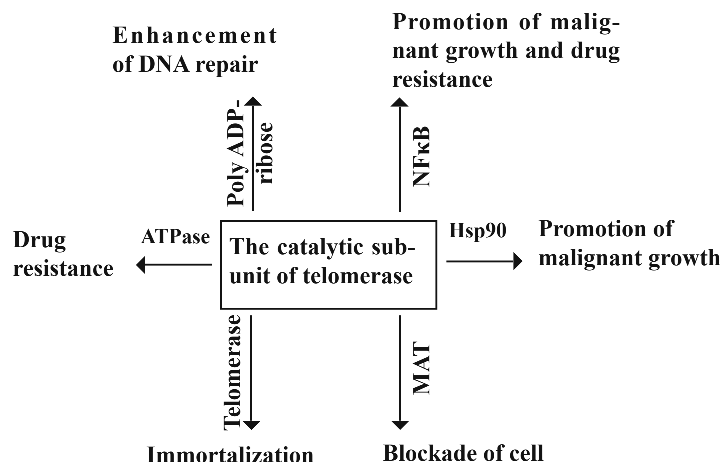


Chart 1. Multiple roles of the catalytic subunit of telomerase in the promotion of malignant growth.

fect[12] on the ternary methylation enzymes to ensure efficient methylation of nucleic acids, so that the hypomethylation of nucleic acids necessary for the cell to undergo differentiation[5] cannot take place. Consequently the differentiation of cells expressing telomerase is always blocked. The blockade of differentiation of normal telomerase expressing stem cells is strategic rather than permanent as in the case of cancer cells. There is an obvious need to build up mass by blocking differentiation for the development of body. When development calls for differentiation, the normal telomerase expressing stem cells can synthesize retinoic acid to unblock the blockade[33,34]. Such a built-in mechanism to unblock the blockade of differentiation is missing in cancer cells. Differentiation inducers to knock out telomerase such as retinoic acid and CDA-2 will be necessary to achieve the induction of terminal differentiation of cancer cells[13].

The identification of the tumor factor of MAT^{LT} as the catalytic subunit of telomerase also helps to explain why telomerase can protect the stability of DNA MT[23], and why telomerase expressing cells can promote cell replication without growth factor[35]. This is because telomerase can stabilize ternary methylation enzymes, and by stabilizing these enzymes they provide the driving force for cell replication.

Two major bands of telomerase were detected in the tumor MAT^{LT} preparations, one with M_r 120 K corresponding to the intact telomerase, and the other with M_r approximately 140-150 k. It is possible that additional protein besides telomerase is involved in the interaction with the tumor MAT^{LT}. It is of interest in this connection to note that the association of telomerase with Hsp90 also involved an additional protein p23[24]. Thus, there is a close analogy in the association of telomerase with MAT and Hsp90. In association with Hsp90, it converts the weak ATPase activity into a very active molecule[25]. MAT also possesses triphosphatase activity very closely related to ATPase. It is possible that the active center of ATPase may play an important role to attract the association with telomerase. The association of MAT with telomerase may be attributable to this similar attraction. If ATPase active center is important to attract the association with telomerase, there is an important question awaiting answer: Will ATPase activity of MDR-1 of drug resistant cancer cells or ABCG2 of cancer stem cells be dictated by telomerase? There must be some close relationship between these two entities, either the two enzymes are closely associated like Hsp90, or they share a structural similarity making them be affected by the same ligand. Therefore, telomerase inhibition can sensitize drug responses[36,37]. By the same token, the reversal of drug resistance by CDA-2[38] may also be attributable to the same factor.

Telomerase once dissociated from the tumor MAT^{LT} becomes very unstable and quickly degraded. We have previously noticed that the proteins recovered in the inactive protein fractions eluted before the active enzyme peak under dissociation conditions of Fig.2a contained the tumor factor which could potentiate the activity of the stripped tumor MAT^{L}, and increase its K_m value. But it could only increase the K_m value to 11 μM, not the native 23 μM, and this biological activity of dissociated tumor factor was no longer detectable a week later[9]. The unstable nature of the tumor factor of MAT^{LT} once dissociated from the enzyme as revealed in this study provides a rational explanation for why the dissociated tumor factor was unable to restore the K_m value to the native state, and for the quick loss of biological activity. Because the tumor factor is so unstable once dissociated from the tumor MAT^{LT}, it is very difficult to study the identity of this tumor factor. The identification of the tumor factor as the catalytic subunit of telomerase also solves the puzzle on the identity of the tumor factor of MAT^{LT}.

It is apparent that the effect of PP-0.39 on telomerase is far more sensitive than its effect on the tumor factor of MAT^{LT}. It required only 30 μg/ml to completely inhibit the telomerase activity in vitro (Table 3), but it required

0.25 mg/ml to eliminate the effect of the tumor factor on the tumor MAT^{LT} to convert it to MAT^{L} [5]. It probably requires much less effort to inhibit enzyme activity than to antagonize protein interaction. It is no surprise that the telomerase was inhibited to a great extent by relatively low dosages of PP-0.39 in cultured HL-60 cells, whereas the induction of differentiation by such low dosages of PP-0.39 could only reach modest levels much lower than the extent of telomerase inhibition. Differentiation inducers that act effectively on the tumor factor of MAT^{LT} such as PP-0.39 are automatically potent inhibitors of telomerase, but not the vice versa. Telomerase inhibitors that act on the telomere DNA or the telomerase RNA are not likely to affect methylation enzymes to achieve the induction of differentiation. Inhibitors of telomerase which are also toxic to prevent cell replication can not induce differentiation either, because such inhibitors cannot cause DNA hypomethylation to activate differentiation genes. Inhibitors must have selectivity to affect only telomerase to become good differentiation inducers. Selective inhibition of the tumor MAT^{LT} as displayed by PP-0.39 in Fig.5 is an appropriate measure to screen for good differentiation inducers. Helenalin is a potent inhibitor of telomerase[39]. Helenalin is, however, very toxic as revealed by its greater inhibition of MAT^{L} than MAT^{LT} (date not shown). Non-selective inhibitors of telomerase such as helenalin may not be able to induce differentiation.

Almost all cancer cells express telomerase, whereas the majority of somatic cells do not. Therefore, telomerase is an ideal target for cancer therapy. If telomerase is only involved in the maintenance of telomere, it is probably not an attractive therapeutic target. Because even if telomerase is totally eliminated, cancer cells without telomerase can still divide for at least 10 generations, which is probably enough to cause fatal damage once the symptom has appeared. Evidently the catalytic subunit of telomerase is involved in many functions other than the maintenance of telomere. Multiple roles of the catalytic subunit in the complexity of cancer is summarized in Chart 1. The catalytic subunit by itself is very unstable. It has to associate with RNA or protein to become stable and functional. It has been shown that the catalytic subunit of telomerase is associated with many enzymes critically involved Chart 1. Multiple roles of the catalytic subunit of telomerase in the promotion of malignant growth in the promotion of malignant growth. Taking cue from its association with Hsp90 to convert the originally weak ATPase activity into a very active enzyme[25], and the association with MAT to transform methylation enzymes into exceptionally stable and active enzymes[1,9], the association of the catalytic subunit of telomerase is very likely to have a positive influence on the enzymes it becomes associated with. Therefore, the catalytic subunit of telomerase is an important factor for the promotion of malignant growth. Thus the elimination of the catalytic subunit of telomerase will have significant impact on cancer therapy. The differentiation therapy offered by retinoic acid and CDA-2 was targeted on the tumor factor of MAT^{LT} [13,14], namely the catalytic subunit of telomerase. Retinoic acid is the drug of choice for the therapy of acute promyelocytic leukemia[40]. Inhibitors of DNA MT are particularly good for the therapy of MDS (myelodysplastic syndrome), which is a disease attributable to the blockade of differentiation of hematopoietic stem cells. Inhibitors of DNA MT 5-azacytidine and 5-azadeoxycytidine were the two drugs approved by the FDA of USA for the therapy of MDS. CDA-2 had comparable therapeutic efficacy as these two drugs, but without toxic side effects displayed by these two drugs[40]. CDA-2 has the potential to become the drug of choice for the therapy of MDS. Another potential application of telomerase inhibitors is on cancer stem cell therapy. Cancer stem cells express a high level of telomerase similar to embryonic stem cells, which plays a dominant role in causing the unique behavior of cancer stem cells. The expression of membrane associated ATPase, namely ABCG2, together with a high level of telomerase is particularly troublesome, because such cells are very resistant to cytotoxic drugs[41]. Radiation cannot hurt these cells either, because most of them are in quiescent state[42]. Besides these cells are equipped with an enhanced ability to repair radiation damage. Inhibition of telomerase is a good approach to attack cancer stem cells. Indeed, CDA-2 has been shown effective in eliminating cancer stem cells[43].

Conflict of interest statement

No potential conflicts of interest were disclosed.

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