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Induction of Cytotoxicity by Hexamethylene Bisacetamide is Associated with the Activity of Telomerase in RBL-2H3 Cells

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RBL-2H3 세포에서 hexamethylene bisacetamide의 세포독성과 telomerase 활성과의 관계

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요 약

본 연구는 비만세포의 일종인 RBL-2H3 세포주에 대한 hexamethylene bisacetamide (HMBA)의 세포독성 유도 기전이 telomerase의 활성과 관계가 있다는 것에 대한 보고이다. RBL-2H3 세포주에 5 mM HMBA를 처리시 그 세포주의 세포성장이 억제 되었다. 기전연구로서 HMBA는 telomerase 활성을 억제하였으며 RT-PCR에 의한 결과 telomerase mRNA 발현의 억제에 의한 것이었다. Madl은 telomerase의 발현을 억제하는 억제인자로 잘 알려져 왔으며 이를 확인해본 결과 Madl의 발현은 유의성있게 증가 되었다. 이를 종합해보면, HMBA에 의한 RBL-2H3 세포주에 대한 세포독성은 Madl 발현의 증가에 따른 telomerase 발현의 억제에 기인하는 것으로 밝혀졌다.

Key words : Hexamethylene bisacetamide (HMBA), Cytotoxicity, Telomerase

INTRODUCTION

Induced differentiation of transformed cells by hybrid polar compounds, such as hexamethylene bisacetamide (HMBA), is regularly associated with a loss of proliferative capacity (Michaeli *et al.*, 1992). This is generally associated with cell cycle arrest in G1 (Marks *et al.*, 1994). In some cell lineages, inducer-mediated loss of proliferation reflects programmed cell death (Williams, 1991).

Telomeres are essential elements that protect chromosome ends from degradation and ligation, contributing to chromosomal stability (Harley *et al.*, 1990; Greider, 1991). Telomeres undergo progressive shortening with cell division because of the inability of DNA polymerase to completely replicate the ends of chromosome DNA (Watson, 1972). The critical shortening of telomeres with cell division induces replicative senescence. Further dividing of cells beyond senescence results in a serious loss of telomeres, causing chromosomal instability. This

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includes end-to-end fusions and dicentric or multicentric chromosomes, and leads to cellular crisis (Van Steensel, 1998). Telomerase is a specialized ribonucleoprotein polymerase that directs the de novo synthesis of telomeric repeats at chromosome ends (Harley *et al.*, 1990). Telomerase is not active in most somatic tissues but is widely activated in cancer cells (Kim *et al.*, 1994; Shay and Bacchetti, 1997). Telomerase activation is thought to be required for cells to continuously divide beyond replicative senescence and may, therefore, be critical step in cellular immortality and carcinogenesis (Counter *et al.*, 1992; Counter *et al.*, 1994).

In this report we address for the first time that induction of cytotoxicity by HMBA in RBL-2H3 cells is associated with the reduction of telomerase activity. HMBA suppressed the expression of telomerase by time and also induced cytotoxicity in RBL-2H3 cells.

MATERIALS AND METHODS

1. Chemicals

Materials were obtained from the following sources: hexamethylene bisacetamide (HMBA) from Aldrich (Metuchen, NJ); telomerase PCR ELISA kit from Roche Molecular Biochemicals (Indianapolis, IN); other reagents for culture of RBL-2H3 cells from Life Technologies or Biofluids (Rockville, MD); RBL-2H3 cells from American Type Culture Collection (Manassas, VA).

2. Cell culture

RBL-2H3 cells were cultured as monolayers in suspension-MEM supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM). All cultures were maintained in humidified atmosphere of 95% air and 5% CO_2 at 37°C.

3. Determination of cell growth

Cell viability was determined by trypan blue exclusion. The cells were harvested and counted in a hemocytometer.

4. Telomerase assay

Telomerase was assayed by use of a telomerase polymerase reaction/ELISA kit. Cells (3×10^5) were collected by centrifugation (3,000 × g, 10 min, 4°C), washed once in ice-cold PBS, and resedimented by centrifugation (3,000 × g, 10 min, 4°C). The pelleted cells were resuspended in 200 μl of lysis reagent as supplied in the kit and left on ice for 30 min. Cell lysates were centrifuged (6,000 × g, 20 min, 4°C), and the supernatant extracts were stored at −80°C until use. The telomeric repeat amplification protocol was performed with extracts containing 1,500 cell-equivalents for each reaction (30-min incubation and 30 cycles). For samples with high telomerase activity, a 15-min incubation and 25 cycles were used. The hybridization and ELISA procedures were performed according to the manufacturer's protocols (Roche Molecular Biochemicals), and the absorbance of the final colored reaction product was measured in a microtiter plate reader at 450 nm. Each arbitrary unit was defined as absorbance per 1,500 cell equivalents.

5. Extraction of RNA and RT-PCR

Total RNA was isolated from RBL-2H3 cells by use of Trizol Reagent (Invitrogen, Carlsbad, CA) and was reverse transcribed with the Superscript first strand synthesis system (Invitrogen) according to the manufacturer's protocol. PCR was performed at 94°C for 45 s, 55°C for 45 s, and 72°C for 60 s for 30 cycles. The following primers were used: 5'-CGGAAGA-GTGTCT-3', reverse 5'-GGATGAAGCGGAGT-CT GGA-3' for telomerase; 5'-GTGGAGTCTAC-TGGCGTCTTC-3', reverse 5'-CCAAGG CTG TGGGCAAGGTCA-3' for GAPDH.

6. Western blotting analysis

The cells were harvested by trypsinization, transferred to 6-well (1×10^6 cells/3 ml/well) cluster

plates, and incubated overnight in complete growth medium with or without 5 mM HMBA. The cells were lysed in 0.25 ml with ice-cold lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1% Nonidet p-40, 10% glycerol, 60 mM octyl β-glucoside, 10 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 2.5 mM nitrophenylphosphate, 0.7 μg/ml pepstatin, and protease inhibitor cocktail tablet). Lysates were kept in ice for 30 min and then centrifuged 15,000 × g for 15 min at 4°C and the supernatant were dissolved in 2x Laemmli buffer. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher and Schuell, BA85). The immunoreactive proteins were detected by use of horse-radish peroxidasecoupled secondary antibodies and Enhanced Chemiluminescence according to the manufacturer's instructions (Amersham Biosciences, Sweden).

7. Expression of results

Unless indicated otherwise, data were expressed as the mean and SEM of mean values from three or more separate experiments. For individual experiments, three to six samples or cultures were assayed for each data point.

RESULTS

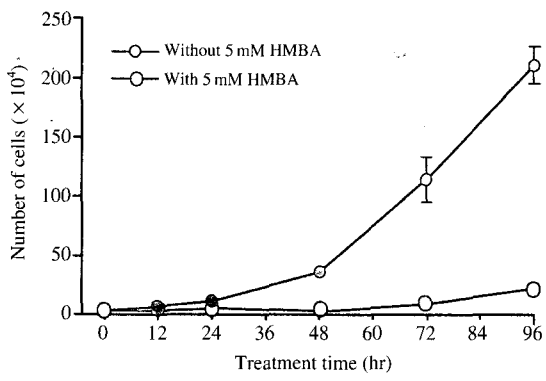
1. Inhibition of cell growth by HMBA

As shown in Fig. 1A, cell growth of RBL-2H3 cells was inhibited by 5 mM HMBA. The activity of telomerase was dramatically reduced to basal level from 24 hr after the treatment of 5 mM HMBA (Fig. 1B). The activity was minimal at 48 hr-treatment with 5 mM HMBA.

2. Down-regulation of telomerase expression by HMBA

To investigate the mechanism of the suppression of telomerase activity, we measured the level of telomerase expression by RT-PCR. Consistent with Fig. 1B, the expression of telomerase was dramatically suppressed 24 hr after the treatment of 5 mM HMBA and was not detectable at 48 hr after the treatment (Fig. 2).

A. Cell growth



B. Telomerase activity

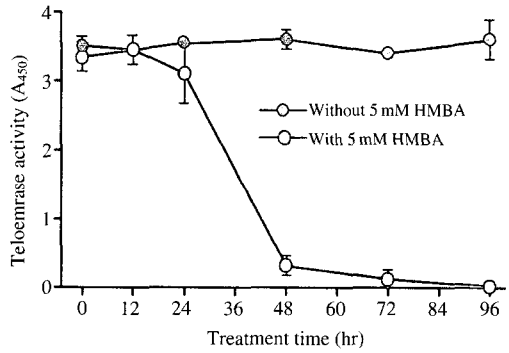


Fig. 1. Cell growth and the activity of telomerase were suppressed by 5 mM HMBA. The cells (2.5 × 104) was plated in 6-well plate, and harvested and counted in a hemocytometer. Cell viability was determined by trypan blue exclusion (A). Cells were incubated with 5 mM HMBA and telomerase was assayed by use of a telomerase polymerase reaction/ELISA kit as in "Materials and Methods" (B).

3. Recovery of cell growth and telomerase activity

Next, we investigated whether there was a correlation between cell growth and telomerase activity by

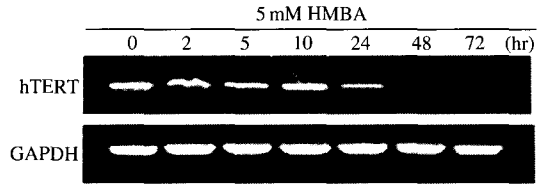


Fig. 2. The expression of telomerase was suppressed by 5 mM HMBA. Total RNA was isolated from RBL-2H3 cells by use of Trizol Reagent (Invitrogen, Carlsbad, CA) and was reverse transcribed with the Superscript first strand synthesis system (Invitrogen) according to the manufacturer's protocol as in "Materials and Methods".

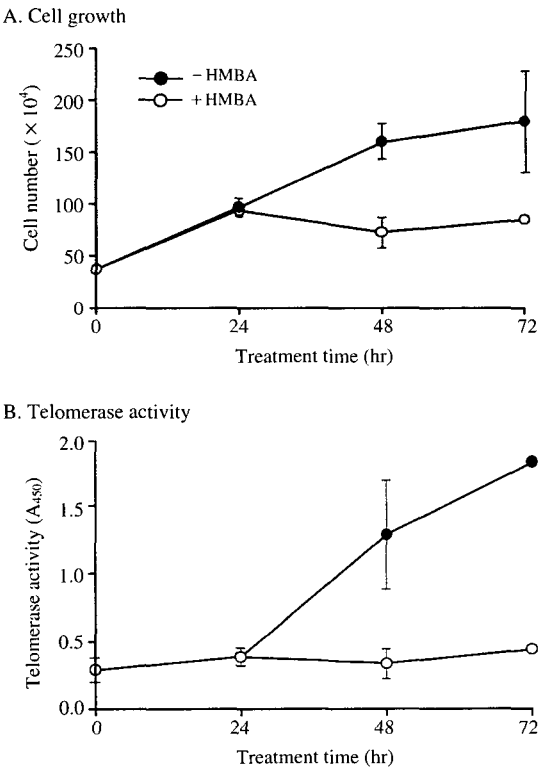


Fig. 3. The recovery of cell growth was correlated with telomerase activity. HMBA was removed 48 hr after the treatment, and cells were continuously cultured. The growth and telomerase activity were measured as in "Materials and Methods".

5 mM HMBA. HMBA was removed from media 48 hr after the treatment and measured cell growth and telomerase activity. The cells restarted to divide 24

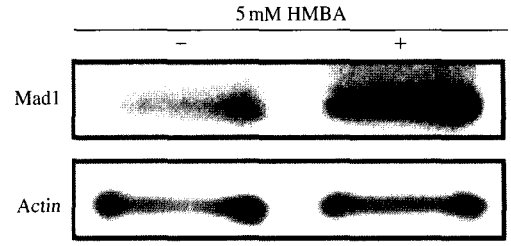


Fig. 4. The repression factor to telomerase expression, Mad1, was induced by 5 mM HMBA. The cells were incubated for 48 hr in 5 mM HMBA and cells were wshed twice with ice-cold PBS. For immunoblotting, cells were lysed in 0.25 ml ice-cold lysis buffer. After centrifugation, the supernatant was dissolved in 2x Laemmli buffer and proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher and Schuell, BA85). The immunoreactive proteins to Mad1 was detected by use of horse-radish peroxidase-coupled secondary antibodies and Enhanced Chemiluminescence according to the manufacturer's instructions (Amersham Pharmacia Biotech).

hrs after removal of HMBA and the telomerase activity showed a same pattern (Fig. 3).

4. Increase of Mad1 expression

To elucidate the mechanism by which HMBA suppresses the activity and expression of telomerase in RBL-2H3 cells, we analyzed the expression of a critical telomerase-related protein, Mad1, which is known to regulate the expression of telomerase. As shown in Fig. 4, the expression of Mad1 was significantly increased by treating 5 mM HMBA after 48 hrs incubation. This result suggest that HMBA could induce suppression of telomerase by down regulating Mad1 in RBL-2H3 cells.

DISCUSSIONS

Dysregulations of cell proliferation, differentiation and apoptosis are hallmarks of cancer cells (Hanahan and Weinberg, 2000). It is possible that these malignant features could be altered by many differentia-

tion inducers such as hybrid polar compound HMBA. Considerable progress has been made toward elucidating the mechanism by which HMBA induces terminal differentiation of cancer cells. In recent year, some reports showed that HMBA induced apoptosis on a number of cancer cells (Siegel *et al.*, 1998; Shelly *et al.*, 1999; Ruefli *et al.*, 2000; Zhang *et al.*, 2000; Hyman *et al.*, 2001), which appeared characteristics of cell shrinkage, chromatin condensation, DNA fragmentation and membrane blebbing (Kroemer *et al.*, 1998; Liu *et al.*, 2002).

In this study we demonstrated that the hybrid polar compound, HMBA, which is a potent inducer of differentiation of varius transformed cells (Michaeli *et al.*, 1992; Marks *et al.*, 1994), inhibited the growth of RBL-2H3 cells and induces morphological and biochemical changes (data not shown), consistent with apoptotic cell death. These included the inhibition of cell growth, telomerase activity, expression of telomerase, and induction of Mad1 expression as the mechanism of suppression of telomerase expression.

To investigate the mechanism by which HMBA induces cellular apoptosis, we studied the effect of HMBA on the activity and expression of telomerase. Telomerase activation is thought to be required for cells to continuously divide beyond replicative senescence and may, therefore, be a critical step in cellular mortality and carcinogenesis (Counter *et al.*, 1992; Counter *et al.*, 1994). The activity and expression of telomerase was dramatically inhibited by HMBA (Figs. 1 and 2). These results strongly suggested that the inhibition of telomerase activity was caused by the downregulation of telomerase in RBL-2H3 cells.

Next, we investigated the mechanism of the suppression of telomerase expression. The only transcription factor that has thus far been implicated in the regulation of telomerase is encoded by the c-myc oncogenes (Greenberg *et al.*, 1999; Greider, 1999; Wang *et al.*, 1998; Wu *et al.*, 1999). The ability of c-Myc to function as a transcription factor has been shown to depend upon its dimerization with the protein Max (Schreiber-Agus *et al.*, 1998). In addition to the formation of stable complexs with c-Myc, Max also heterodimerizes with proteins of the Mad family (Ayer *et al.*, 1993; Zervos *et al.*, 1993). These Mad/Max complexes act in an antagonistic manner to c-Myc/Max-induced transactivation and results in potent repression of gene expression. Based on the reports, we measured the expression level of Mad1 by HMBA. The expression of Mad1 was significantly increased by HMBA (Fig. 4). This result suggested that the suppression of telomerase activity was induced by Mad1 increased by HMBA.

In conclusion, HMBA suppressed the proliferation of RBL-3H3 cells and induce cellular toxicity by suppressing telomerase activity, probably, through Mad1/Max complex.

ACKNOWLEDGMENTS

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