CLONING AND EXPRESSION OF A GENE ASSOCIATED WITH HL_{60} CELL APOPTOSIS INDUCED BY INHIBITION OF POLYAMINE BIOSYNTHESIS

FENG Lixin 冯立新, FAN Muzhen 范慕贞

Department of Biochemistry and Molecular Biology, China-Japan Friendship Institute of Clinical Medical Sciences, Beijing, 100029, China

Abstract

Objective: To clone the gene associated with apoptosis induced by an inhibitor of polyamine biosynthesis, α-difluoromethylornithine (DFMO). Methods: The differential subtraction screening was used for gene cloning from cDNA library of HL_{60} cells treated by DFMO. Northern blot, morphological observation, FCM assays and ladder map of DNA electrophoresis were performed. Results: The transfecting gene expression and activity of inducing apoptosis in the cells transfected from recombinant plasmid containing the cloned fragment df4 was proved. Conclusion: It is suggest that df4 gene cloned in the study could be a gene regulating apoptosis of HL_{60} cells.

Key words: Polyamines, HL_{60} cell, Apoptosis, Gene expression, DFMO

To search whether some blocked genes of tumor cells are repromoted in the events associated with induction of cell differentiation and apoptosis by inhibition of polyamine biosynthesis, screening and cloning of the genes from cDNA library constructed with HL_{60} induced to differentiation and apoptosis by inhibitor of polyamine biosynthesis, DFMO, and the primary analysis of cloned gene expression and activity of apoptosis induction were performed in this study.

MATERIALS AND METHODS

Cell Culture

HL_{60} cells were cultured in PRMI 1640 containing 10% FBS at 37°C and with 5%CO_2. 6mmol/L DFMO was used to induce differentiation and apoptosis of HL_{60} cells. Cellular growth was monitored by cell counts day to day and cellular morphology was observed by Geimsa staining.

DNA Ladder Analysis by Gel Electrophoresis (as routine procedure)

HL60 cell pellet was lysed with lysate buffer (50mmol/L Tris-HC1, 10mmol/L EDTA, 0.5%SDS and Proteinase K) at 60°C for 2 hours, then cooled down to room temperature, extracted with equal volume of Phenol and Phenol/Chloroform, respectively.

The DNA was precipitated from aqueous phase with 0.1 volume of 3mol/L NaAc and 2 volume of Ethanol and dissolved in TE buffer after drying DNA pellet in a vacuum, then fractionated by electrophoresis on 1.6% agarose gel.

Construction of cDNA Library

A cDNA library was constructed as described in reference 8. Briefly, the cDNA was synthesized using Xba I-primer-adapter from total RNAs of HL60 cells induced with DFMO, then methylated with EcoR I-methylase and ligated with phosphorylated EcoR I linker at the opposite end of Xba I site. The cDNA fragment digested with EcoR I/Xba I was ligated to Lambda GEM-4 phage arm and packaged. These packaged particles infected the bacterial host LE392.

Screening and Cloning of the Gene Associated with Induced Differentiation

The antisense cDNA was synthesized from mRNA of differentiated HL60 cells induced with DFMO by reverse transcriptase with substrate mixture containing ^{32}P-dCTP. The labeled cDNAs were purified through G50 column, hybridized with mRNA from parental HL60 cells. The nonhybridized antisense cDNAs as differential subtraction probe were isolated from hybrids by hydroxylapatite column and eluted with 0.16 mol/L Na_3PO_4 from column. Its specific radioactivity was determined.

Screening of positive clone (df4) was performed by routine procedure including plating diluted solution of packaged cDNA library, transferring of DNA from plated plaques to NC filters

Accepted for Publication: January 28, 1999

Correspondence to: Fan Muzhen, Department of Biochemistry and Molecular Biology, China-Japan Friendship Institute of Clinical Medical Sciences, Yinghua East Road, Hepingli Street, Beijing 100029, China; Phone: (0086-010)-64221122 (Ext) 4437; Fax:: (0086-010)-64206643; E-mail: muzhfan@public.east.cn.net

and hybridizing with 32P-differential subtraction probe and autoradiography.

Construction of Eukaryotic Expression Plasmid Containing Interest Insert[8]

The DNA was isolated from amplified positive phage clones. The EcoRI/Xba I digested DNA fragment was ligated into the prepared arm of eukaryotic expression vector pRC/CMV digested with EcoR I/Pst I and Xba I/Pst I, respectively. The recombinant plasmid infected bacteria HB101. From amplified HB101 the recombinant plasmid pCMV/df DNAs was isolated and the inserts were analyzed by restriction endonuclease map.

Screening and Cloning of Transfected Cells

HL60 cells were transfected with recombinant expression plasmid pCMV/df via calcium phosphate coprecipitation.[8] Cell clones pCMV/df-HL60 were selected with G418.

Northern Blot

It was performed as routine procedure with ^{32}P-df4 DNA fragments as probe.

Detection of Cellular Apoptosis

It was carried out by cell morphology, DNA ladder map analysis and FCM.

RESULTS

Construction of cDNA Library of HL_{60} induced to differentiate by DFMO

The size of cDNA Library constructed as described in Materials and Methods was determined by titer detection to be 2.85×10^6 clones/μg mRNA. It should be large enough to screen interest sequences.

Screening of Positive Clones

About 3×10^4 phage clones were transferred onto 30 pieces NC filters and hybridized with ^{32}P-differential subtraction probe. The positive clones were selected by autoradiography. 23 of positive candidate clones obtained from second selection were spotted on two pieces of filters, which were hybridized with wild HL_{60} and induced HL_{60} cell DNA probes, respectively. The results indicated that 9 clones, named as df1-df9, were confirmed to express only in HL_{60} cells induced by DFMO, but not in wild HL_{60} cells (Figure 1).

Construction of Eukaryotic Expression Plasmids pCMV/dfs

The construction of eukaryotic expression plasmid pCMV/dfs was carried out as described in Materials and Methods. The size of df1-df9 inserts was distribution in range of 1.7-2.3kb on gel electrophoresis. The size of insert in pCMV/df4 was 2.1 kb.

Cellular Growth Inhibition and Apoptosis Induction of Transfected HL_{60} Cells by pCMV/df4

The HL_{60} cells were transfected by pCMV/df1-pCMV/df9, respectively. From which the growth of transfectant HL_{60} with pCMV/df4 was suppressed markedly (Figure 2).

Cell morphological observation by light microscopy with Giemsa staining showed that a lot of pCMV/df4 transfected cells underwent apoptosis such as chromatin condensation, margination and fragmentation occurred (Figure 3).

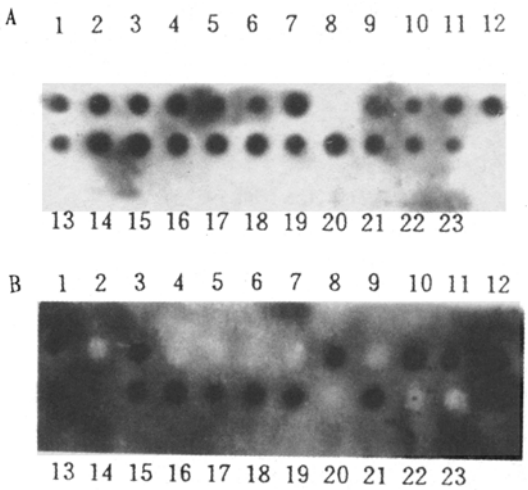

Fig. 1. Dot hybridization of positive clones
A. with cDNA of HL_{60} induced to differentiate
B. with cDNA of HL_{60}

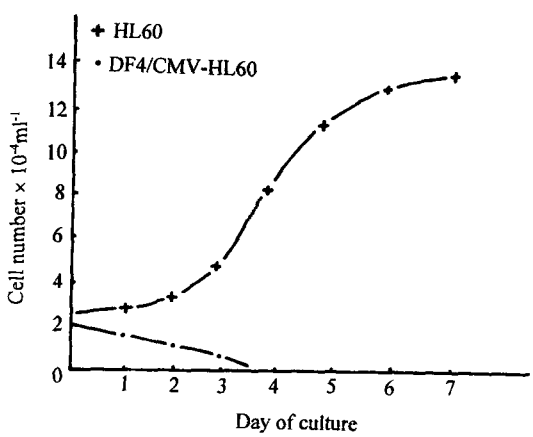

Fig. 2. Growth curve of pCMV/df4-Hl_{60} cells

The fragmentation of transfectant DNA as 200 bp and redoubled fragments as ladder map on agarose gel electrophoresis was detected (Figure 4).

FCM analysis of transfectant cells showed that the apoptosis peak increased obviously with extension of transfection time.

Northern Blot showed that df4 gene expression was observed in pCMV/df4-HL60 cells. (Figure 5)

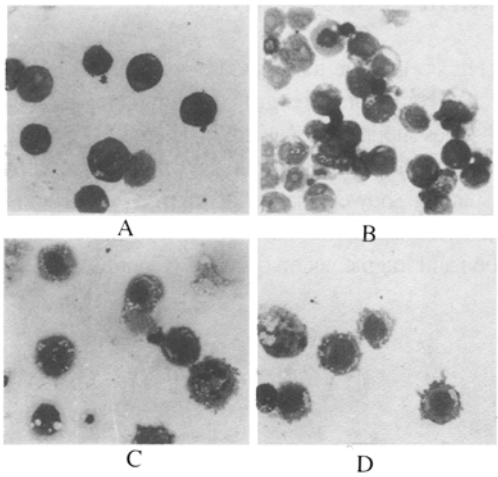

Fig. 3. Cell morphology
A. HL_{60} B. pCMV/df4-HL60 cells (96 hr)
B. PCMV/df4-HL60 cells (148 hr)
C. pCMV/df4-HL60 cells (172 hr)

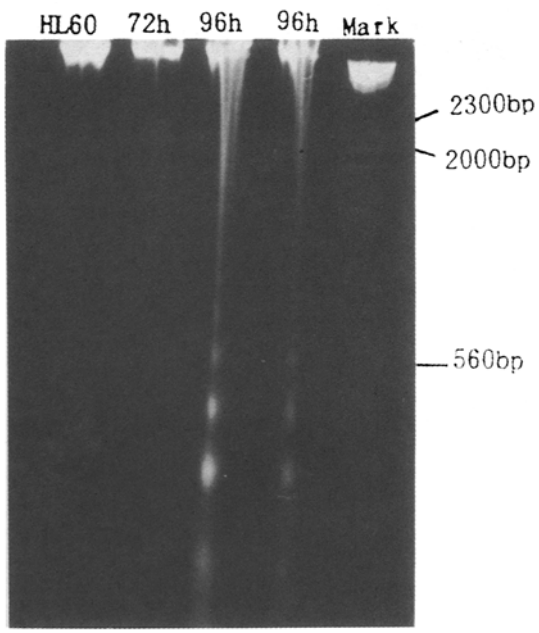

Fig. 4. DNA electrophoresis of pCMV/df40HL60 cells

DISCUSSION

The regulation of polyamine metabolism plays an important role in cell proliferation and differentiation.[1] The malignant transformation of normal cells is closely related with anomaly polyamine metabolism.[2] In our previous study it was showed that DFMO, an inhibitor of ODC which is a key

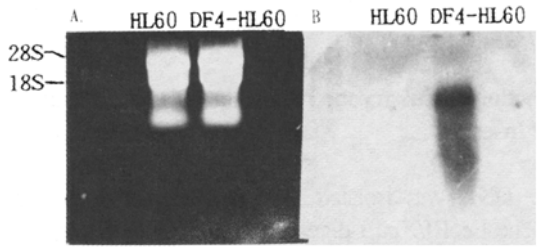

Fig. 5. Northern hybridization of pCMV/df4-HL60 cells
A. RNA electrophoresis
B. Northern blot

enzyme of polyamine biosynthesis, could inhibit the growth proliferation, induce differentiation and apoptosis of tumor cells and elicit the decreasing or losing of tumorigenicity. Its mechanism should be involved in regulation of associated gene expression.[3-6] It was observed that the phenotypes of Ha-ras^{val12} transformed cells HR-I were reversed by treatment with DFMO, but this reversion was prevented when exogenous putrescence was given along with DFMO.[7] These results have elucidated that polyamines are an important element in malignant transformation and maintenance of its phenotypes; the inhibition of polyamine biosynthesis is a key target of tumor reversion. To screen the gene associated with cell differentiation and apoptosis induced by DFMO, the cDNA library of differentiation HL60 cells induced by DFMO was constructed. Using differential subtraction screening, 9 cDNA (df1-df9) clones were isolated from the library. Transfection experiments with recombinant eukaryotic expression plasmids containing df1-df9, respectively, showed that df4 resulted in cell growth arrest and apoptosis, although no change in growth rate of transfectants with other[8] clones was observed. It is suggested that df4 could be a gene sequence associated with cell apoptosis, but the possibility is not ruled out that simultaneous expression of several other clones could regulate cooperatively cell growth and differentiation. It should be further studied, whether expression of these genes are promoted sequentially and regulate the expression of associated oncogenes and/or antioncogenes, effects on the maintenance of tumor malignant phenotype, thereby the reversion of malignant phenotype occurs. Therefore, the cloning of the genes associated with differentiation and apoptosis induced by inhibition of polyamine biosynthesis and study on its structure and function are an important project to elucidate the molecular mechanism of tumor malignant phenotype maintained by polyamines and reversed by the inhibition of polyamine biosynthesis.

REFERENCES

[1] Pegg AE. Polyamine metabolism and its importance in neoplastic growth and as a target for chemotherapy. Cancer Res 1988; 48:759.

[2] Moshier JA, Dosescu J, Skuncam, et al. Transformation of NIH/3T3 cells by ornithine decarboxylase overexpression. Cancer Res 1993; 53: 2618.

[3] Fan Muzhen, Qi Shangle, Zhao Chenglong, et al. Effects of

DFMO on the cell growth, Differentiation and expression of c-myc, e-fos gene of HL60 cells. Progress in Biochemistry and Biophysics 1989; 16: 455.

[4] Wu Kebin, Fan Muzhen. Effects of difluoromethylornithine on the expression of c-myc, c-fos, c-Ha-ras and ODC gene in HL60 cells. Journal of China-Japan Friendship Hospital 1992; 6 (Suppl): 224.

[5] Chen Shiming, Zhao Chenglong, Wu Hui, et al. Establishment of human promyelocytic leukemia xenograft model and its experimental treatment. Journal of China-Japan Friendship Hospital 1992; 6: 68.

[6] Feng Lixin, fan Muzhen. Induction of differentiation and Apoptosis of HL60 cells with DFMO. Journal of China-Japan Friendship Hospital 1996; 10: 121.

[7] Wang Jinqing, Fan Muzhen. The regulation of the inhibition of polyamine biosynthesis to the expression of c-Ha-ras oncogene in transformed cells. Chin J Biochemistry 1995; 11: 297.

[8] Sambrook J, Fritsch EF, Maniatis T. Molecular cloning, a laboratory manual. 2nd Ed. New York: Cold Spring Harbor Lab Ppress, 1989; 16: 17–29.