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Basic Investigations

EXPRESSION OF c-myc GENE AND BIOSYNTHESIS OF BIOLOGICAL MACROMOLECULES IN ANTISENSE TRANSFECTANT HL^R_{60} — 9*

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The recombinant plasmid PGC was constructed for transcription unit of c-myc gene with diorientation *in vitro*, to make RNA probes for detection of c-myc mRNA and antisence RNA expression of tranfectant HL^R_{60}-9, which was obtained from HL60 cells transfected with inducible c-myc antisense RNA expression plasmid. The results from HL^R_{60}-9 cells induced by Cd^{2+} indicated that expression of c-myc antisense RNA increased with Cd^{2+} concentration and exposure time, while c-myc mRNA expression progressively reduced. Using immunohistochemical technique no c-myc P62 protein expression was detected. The incorporation of ^{3}H-TdR, ^{3}H-UR and ^{3}H-Leu revealed significant suppression of DNA 、 RNA and protein biosynthesis. It is suggested that the reversion changes previously reported in malignant phenotypes of HL^R_{60}-9 cells and the inhibition of macromolecular biosynthesis mentioned above were associated with the blockade of c-myc gene expression by its antisense RNA.

Key words: c-myc antisense RNA , Gene expression, DNA biosynthesis, RNA biosynthesis, Protein biosynthesis

In our previous study HL60 cells were transfected with the inducible c-myc antisense RNA expression plasmid constructed by us.[1] Introduction of c-myc antisense RNA expression plasmid to HL60 cells resulted in the changes in growth characteristics of the transfectant HL^R_{60}-9 such as inhibited cell growth, precluded entry of cells from G1/G0 to S state, induced differentiation of cell morphology and function and markedly reduced tumorigenicity. It indicated that specifically controlling c-myc expression by antisense methodology is a available pathway for reversing malignant phenotype of tumors. To confirm that these changes are associated with blocked c-myc expression by it antisense RNA, in this report the recombinant plasmid containing transcription unit of c-myc gene with diorientation was constructed to make the probes *in vitro* for detection of c-myc mRNA and antisense RNA expression in antisense transfectants and the effects of controlling c-myc gene expression on biosynthesis of biological macromolecules DNA 、 RNA and proteins were observed.

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MATERIALS AND METHODS

Plasmid PGEM-3Z (2.7Kb)

It was purchased from Promega, USA. pMC41-c-myc (12.3Kb) provided generously by Dr. Land H. Restriction endonuclease, T4 DNA ligase, T7 and Sp6 RNA polymerases were purchased from Sino-American Biotechnology Co.; α-^{32}P-dCTP from Fu-Rui Co.; ^{3}H-TdR, ^{3}H-UR and ^{3}H-Leu from amersham, UK.

Plasmid pGXC

It was constructed in our Lab,[1] which is a c-myc antisense RNA expression plasmid inducible by Cd^{2+} in eukaryotes.

The Procedures for Preparation of Plasmids, Recovery of DNA, DNA Ligation and Trans--formation of Bacterium

They were as described in "Molecular Cloning" edited by Sambrook J., et al.[2]

Cell Culture and Transfection

It was carried out as described in a earlier report.[1] The plasmid pGXC and pSVneo were cotransfected into human promyelocytic leukemia cells HL60 via electroporation. Several colonies of transfectants HL^{R}_{60} were selected for resistance to G418. HL^{R}_{60}-9 from them was taken as study model.

Transcription *in vitro* of the Plasmid Containing Transcription Unit with Diorientation (Preparation of RNA Probes)

To the solution of linear DNA (1 μg/μl) was added 2μl 10×buffer (400 mmol/L Tris-HC1, pH7.2, 60 mmol/L $MgC1_2$, 20 mmol/L spermidine, 50 mmol/L NaC1), 0.5 mmol/L ATP, UTP, CTP, 20 μ RNasin, 50 μ Ci/5 μ 1 α-^{32}P-GTP(3000Ci/mmol), 10 μ T7 or Sp6 RNA polymerase, and sterile ion-free water treated with DEPC was added to make final volume to 20 μl. This reaction tube was incubated at 37 ℃ for 2 hrs. 1 μ 1 of RNAase-free DNase I (1 mg/ml) was added and incubated at 37 ℃ for another 15 min., followed by addition of 10 μg tRNA and 100 μl water treated with DEPC. This solution was extracted for thrice with phenol/chloroform and precipitated by ethanol according to routine procedure. The precipitated pellets was dissolved in 20 μl of DEPC-treated water as RNA probe.

Slot Blot

The detected RNA was spotted on a nylon sheet (genescreen plus, USA) using the apparatus (minifold II Schleicher and schuell, Germany) for spotting sample, hybridized with RNA probe and exposed to film by procedures as same as that for dot blot described in reference.[2]

[^{3}H] Incorporations

It was performed by the method as described previously[3] for determination of DNA 、 RNA and protein biosynthesis.

Immunohistochemical Analysis for c-myc P62

It was carried out using ABC kit (Vector Lab, USA) by routine procedure. Monoclonal antibody to c-myc P62 was from Shenyang Medical university.

RESULTS

Construction of Expression Plasmid pGC Containing C-myc Transcription Unit with Diorientation

A 2.7Kb XbaI/BamHI restriction fragment from plasmid pGEM-3Z was as recombinant plasmid vector. A 1.85Kb restriction fragment containing c-myc intron 1-exon 2 sequence was excised from plasmid PMC41-c-myc by digestion with XbaI/Bg1II and inserted into pGEM-3Z between Sp6 and T7 promoters at XbaI/BamHI site. 1.85 Kb c-myc antisense RNA and mRNA fragment were obtained by transcription of the insert using T7 RNA polymerase and Sp6 RNA polymerase, respectively, as sense and antisense probes. The verification of plasmid construction was performed by digestion with restriction endonuclease followed by Southern blot analysis (Figure 1).

C-myc mRNA and Antisense RNA Expression in Transfectant HL^{R}_{60}-9

Slot blot followed by hybridization with ^{32}P-

sense RNA probe showed that the expression of c-myc antisense RNA was detected in HL^{R}_{60}-9 cells at 4 hr after Cd^{2+} induction, increased with extension of induction time (Figure 2A) and related positively to Cd^{2+} concentration used in induction. On the contrary,hybridization with ^{32}P-antisense RNA probe indicated that c-myc mRNA expression in HL^{R}_{60}-9 cells reduced progressively with Cd^{2+} induction time (Figure 2B). Black density scanning showed the changes in sense and antisense expression with time as mirron image each other (Figure 3)

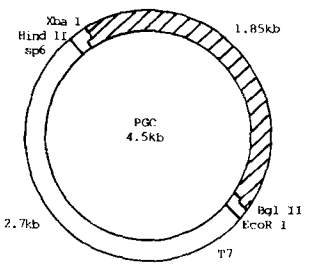


Fig 1. Construction of plamid PGC

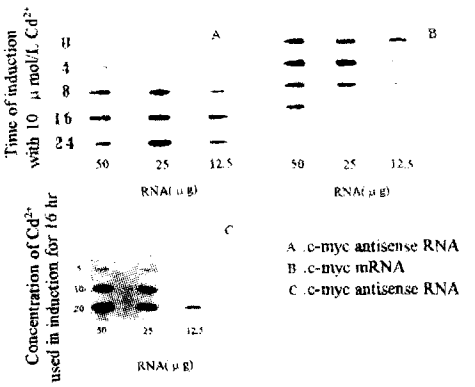


Fig 2. Expressions of c-myc antisense RNA and mRNA induced by Cd^{2+}

C-myc P62 Protein Expression in HL^{R}_{60}-9 Cells

A marked inhibition of c-myc P62 expression in HL^{R}_{60}-9 cells induced with 10 μ mol/L Cd^{2+} was found by immunohistochemical analysis in comparison to control cells (Figure 4).

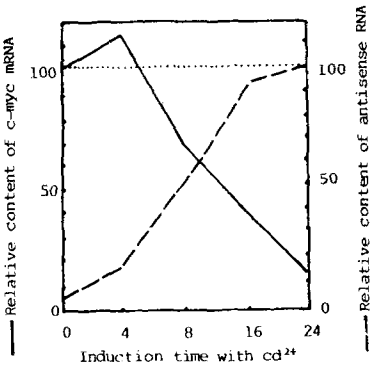
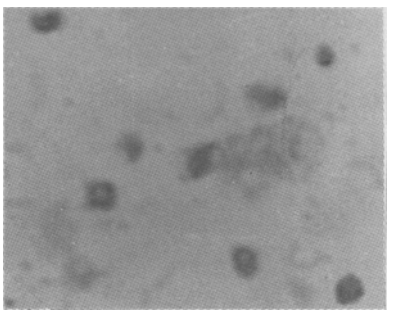


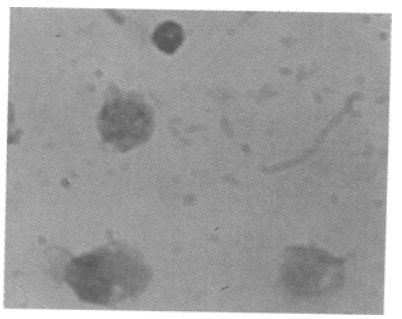
Fig 3. Relativity of c-myc antisense RNA and mRNA expressions in HL^{R}_{60}-9 cells

Biological Marcromolecular Biosynthesis in HL^{R}_{60}-9 cells

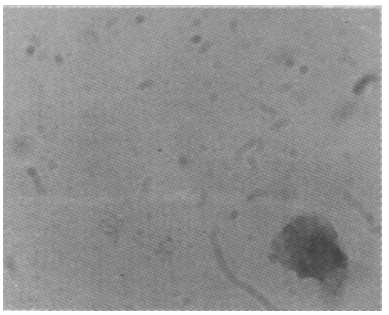
^{3}H-thymidine, ^{3}H-uridine and ^{3}H-leucine incorporation revealed that c-myc antisense RNA expression induced by Cd^{2+} in HL^{R}_{60}-9 resulted in obvious inhibition of DNA 、 RNA and protein biosynthesis to 12.5%、21.0% and 11.0% of parental cells HL60, respectively (Figure 5).



A. HL60



B. HL^{R}_{60}-9



C. HL^{R}_{60}-9 + 10 μ mol/LCd^{2+}

Fig 4. Inhibition of c-myc P62 protein expression in induced HL^{R}_{60}-9 cells

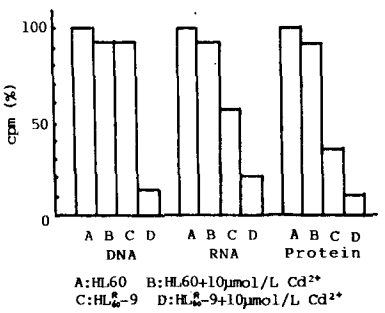


Fig 5. Biosynthesis of DNA 、 RNA and protein in HL^{R}_{60}-9 cells induced by Cd^{2+}

DISCUSSION

The c-myc nuclear oncoprotein plays a important role in the regulation of cell proliferation and differentiation.[4] The enhanced expression of c-myc gene is implicated in genesis of various neoplasias.[5] It was reported that high constitutive expression of c-myc blocks the differentiation of mouse erythroleukemic cells into cells that resemble mature erythrocytes.[6] It has been shown in several cell systems that repression of c-myc expression occurs concomitantly with terminal differentiation. Therefore, controlling c-myc over expression using antisense methodology would be a efficient pathway for regulating the balance between proliferation and differentiation of malignant cells. Our previous studies demonstrated that the growth of HL^{R}_{60}-9 cells transfected by inducibel with Cd^{2+} expression plasmid containing c-myc antisense RNA was inhibited, while morphological and functional differentiation induced. It resulted in reduce or lose of tumorigenicity.[1] The present data show that c-myc antisense RNA expression induced by Cd^{2+} in transfectants is accompanied with inhibition of c-myc mRNA expression. It is revealed that the reversion of malignant phenotypes mentional above is associated with blocked c-myc mRNA and protein expression by c-myc antisense RNA. There is evidence to suggest that the potential mechanisms by which c-myc gene may regulate cell growth involve its activity as a sequence-specific transcriptional regulator;[7] its ability to bind growth suppressors such as Rb gene product;[8] or its ability to induce DNA replication.[9] Our results also indicate that c-myc antisense RNA expression inhibits effeciently the biosynthesis of DNA 、 RNA and proteins in tumor cells, subsequently influence the growth rate of tumors. It is possible that the effects of c-myc expression on cell growth and differentiation may be mediated by a series of reactions, including induction of ODC (a key enzyme in polyamine biosynthesis) expression,[10] activation or inhibition of associated oncogene and antioncogene expressions. Alter-natively, the inhibition of c-myc gene expression by its antisense sequence can preclude certain reactions which are advantageous to tumor cell growth, thereby the reversion of malignant phenotypes occurs.

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