Deguelin Represses Both the Expression of Nucleophosmin and some Nucleoporins: Nup88 and Nup214 in Jurkat Cells

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Since the first report about cytoplasmic nucleophosmin (NPM) in acute myelogenous leukemia with a normal karyotype was announced, the shuttling activity of NPM and its proper subcellular localization have drawn many attentions. Mechanisms that regulate nucleocytoplasmic transport of proteins may provide novel opportunities for drug development. Here we show that, in Jurkat cells, strong fluorescence density of NPM prevails in the nucleus, while, some key nucleoporins: Nup88 and Nup214 localize mainly in the cytoplasm. Deguelin, a natural occurring rotenoid, presents powerful anti-leukemia effects through proliferation inhibition and apoptosis induction in Jurkat cells. Deguelin downregulates the expression of NPM, Nup88 and Nup214 in a dose-dependent manner and reverts the localization of Nup88 and Nup214 to nuclear rim. These results suggest that deguelin exhibit its strong anti-leukemia effects might through the regulation of some nucleoporins, thus influence the subsequent abnormal expressions or localizations of some key proteins involved in proliferation and/or apoptosis, such as: NPM.

Key words deguelin; nucleophosmin; nucleoporin; apoptosis; leukemia

Nucleophosmin (NPM) was first identified as a nucleolar phosphoprotein expressed at high levels in the granular region of the nucleolus.1) NPM has been proved to be a multifunctional protein that is involved in several cellular processes, including ribosome biogenesis, centrosome duplication, cell cycle progression, cell growth, and transformation.2) NPM is implicated in human tumorigenesis, but the function of NPM in tumorigenesis is much confusing, it might be related to both oncogenic and tumour-suppressing pathways, depending on its dosage and level of expression. The NPM gene (NPM1) locus is involved in chromosomal rearrangements, such as translocations or deletions in many kinds of haematological malignancies.3) Strikingly, NPM has also recently been found to be mutated and aberrantly localized in the cytoplasm of leukemia blasts in a high proportion (around 35%) of patients with acute myeloid leukemia (AML), which makes NPM1 one of the most frequently mutated genes in AML.4)

Although the bulk of NPM resides in the granular region of the nucleolus, NPM shuttles continuously between the nucleus and cytoplasm.5) The shuttling activity of NPM and its proper subcellular localization might be crucial for cellular homeostasis. Nuclear import and nuclear export mechanisms that govern the nucleocytoplasmic distribution of macromolecules are regulated at multiple levels. However, nuclear pore complex (NPC), as the only gateway, might produce a marked effect. The NPC is composed of multiple copies of proteins called nucleoporins. In vertebrate cells, about 30 nucleoporins have been identified.6) However, the function of many individual nucleoporins in different transport pathways is largely unresolved. Nup88, a protein is putatively involved in nuclear-cytoplasmic transport and localized preferentially at the nuclear membrane, is found to be overexpressed in a series of tumor cell lines and primary human solid tumors.7—9) CAN, also called Nup214, is a nuclear pore complex (NPC) protein that interacts at the NPC with Nup88. Nup214 was first identified as a target for chromosomal translocations involved in leukemogenesis, and has also been suggested to be involved in proteins transport, nuclear mRNA export, and cell cycle regulation.10,11) Furthermore, variation in levels of the Nup88/Nup214 complex influences the relative strength and duration of NF-kB signaling responses.12) Since the important roles in the nucleocytoplasmic transport and tumorigenesis, Nup214/Nup88 complex might also contribute to the transport of NPM or some other key proteins involved in the genesis or development of leukemia. Mechanisms that regulate nucleocytoplasmic transport of proteins may provide novel opportunities for drug development.

Deguelin, a rotenoid isolated from several plant species including Mundulea sericea (Leguminosae) has been shown to be a strong anti-tumor and cancer chemopreventive agent through PI3K/Akt pathway inhibition in several mammalian cell culture systems.13,14) Our earlier studies have also shown that deguelin presents its powerful antileukemia activity through the modulation of cell cycle regulative proteins or NF-κB signal pathway in vitro.15,16) However, the effect and mechanism of action of deguelin on T cell leukemia is not well known. It is our interest to investigate whether the antitumor activity of deguelin on T cell leukemia is association with the blockade of constitutively active of nucleoporins: Nup88 and Nup214, thus influences the subsequent nucleocytoplasmic transport of shuttling proteins, such as: NPM.

MATERIALS AND METHODS

Materials Jurkat cell line is obtained from the China Center for Typical Culture Collection (Wuhan, China) and maintained in RPMI-1640 (Sigma) medium supplemented with 10% fetal bovine serum (FBS, Gibco-BRL, U.S.A.) at 37 °C in a humidified atmosphere. Deguelin is purchased from the Alexis (Läufelfingen, Switzerland). Anti-NPM antibody is purchased from Zymed, San Francisco, Calif. Antibodies specific to Nup88, Nup214, GAPDH and HRP-conjugated secondary antibodies are all from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). MTT is purchased from Janssen Chimica Company (New Brunswick, New Jersey,
U.S.A.), DNA apoptosis ladder kit is from Boehringer-Mannheim (Germany), Annexin-V FITC detection kit is from BD Biosciences (San Diego, CA, U.S.A.). Chemiluminescence (ECL) reagent kit is purchased from Pierce Biotechnology (Rockford, IL, U.S.A.). Trizol reagent is from Invitrogen Company, U.S.A., RT-PCR kit is purchased from Fermentas, Lithuania, U.S.A.

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyldetrazolium Bromide (MTT) Assay The antiproliferative effect of deguelin against Jurkat cells is determined by MTT method. Briefly, the Jurkat cells are incubated with various concentrations of deguelin for 0, 12, 24, 48, 72 h in triplicate in 96-well plate. Thereafter, 20 μl MTT solution is added to each well. After continued incubation for 4 h, the supernatant is discarded and 150 μl of dimethyl sulfoxide (DMSO) is added. When the blue crystals are dissolved, the optical density (OD) at 570 nm is read with a 96-well multispec scanner autoreader (Biotech Instruments, NY, U.S.A.). The following formula is used: cell proliferation inhibited (%)=\[1-(\text{OD of the experimental samples/OD of the control})\]×100%.

DNA Fragmentation Assay DNA is isolated according to the method described in the DNA apoptosis ladder kit. Cells are washed and lysed in 300 μl of digestion buffer after 24 h and/or 48 h culture. The genomic DNA is dissolved in TE buffer and 5 μg of DNA samples is subjected to agarose gel (2%) electrophoresis. The gels are visualized and photographed under UV light.

Annexin-V Fluorescein Isothiocyanate/Propidium Iodide (FITC/PI) Staining for Evaluation of Apoptosis According to the method described in Annexin-V FITC detection kit, cells are washed and resuspended in the binding buffer provided in the kit after 24 h culture. Afterwards, cells are stained with 5 μl Annexin-V FITC solution and 10 μl PI solution for 15 min at room temperature in the dark. Finally, the samples are diluted with 300 μl binding buffer and analyzed on a flow cytometer (FACSort; BD Biosciences) within 30 min.

Hoechst 33258 Staining Nuclear fragmentation is visualized by Hoechst 33258 staining of apoptotic nuclei. The apoptotic cells are collected, washed, and then fixed in 4% paraformaldehyde for 10 min at room temperature before deposition on polylysine-coated coverslips. The adhered cells are permeabilized with 0.1% Triton X-100 for 5 min at room temperature, and then incubated with Hoechst 33258 for 30 min at 37 °C, rinsed with PBS and mounted on slides with glycerol-PBS. Finally, the cells are viewed with an Olympus BH-2 fluorescence microscope (Tokyo, Japan).

Preparation of Cell Lysates and Western Blot Analysis After treatment, Jurkat cells are harvested and lysed in 100 μl of lysis buffer by incubation on ice for 30 min, then, the extracts are centrifuged at 12000 rpm for 15 min at 4 °C. After addition of sample loading buffer, protein samples are electrophoresed on a 12.5% sodium dodecylsulfate (SDS)-polyacrylamide gel and then transferred onto nitrocellulose membranes, probed with anti-β-actin and anti-Nup88 antibodies (dilution 1:1500) separately. After overnight incubation at 4 °C, the blots are washed, and exposed to HRP-conjugated secondary antibodies (dilution 1:1500) for 1 h. Finally, the blots are washed and detected with ECL substrate solution. Densitometric analysis is performed using Quantity One software.

Nup214 Protein Analysis Using Flow Cytometry Flow cytometry is performed to determine the expression of Nup214 in Jurkat cells. A total of 1×10⁶ cells are collected and washed after 24 h culture, anti-Nup214 antibody (dilution 1:100) is added, the mixture is then kept at 4 °C overnight. Mouse IgG1 (dilution 1:50) antibody is the isotype control group. FITC-labeled secondary antibody (diluted 1:100) is applied for 30 min at room temperature. Stained cells are analyzed on a flow cytometer. The percentage of cells is determined by the CellQuest software program.

RNA Isolation and Reverse Transcription-PCR Cells are lysed within Trizol reagent after 24 h culture, and total RNA is prepared. cDNA was synthesized according to the manufacturer’s instruction of Fermentas kit. Twenty microliters PCR reaction mixture is amplified. The following primer pairs are designed from human cDNA sequences available in GenBank and synthesized by Shanghai Invitrogen Biotechnology Co., Ltd., China: Nup88 (GenBank accession no. NM_002532); 5'-GGAGCTTGCTTTGAAACTGG-3 and 5'-ATTTCGCCGACAGTTCCTC-3; Nup214 (GenBank accession no. NM_005085): 5'-AGTCCTCACGTCCTGGCCCTCA-3 and 5'-CGATTGTTGGCTAGGGTGTT-3; NPM (GenBank accession no. NM_002520): 5'-GTTCAGGGCCAGTGCATATT-3; 5'-ACCTCCTCAGCTGCCAGAGA-3; β-actin (GenBank accession no. NM_001101U), 5'-CTGTCCCTGTATGCTCTG-3 and 5'-ATGTCACGCCAGATTCC-3. After amplification, 8 μl aliquots of products are resolved on a 1.5% agarose gel. DNA bands are quantified by Smart View Bio-electrophoresis Image Analysis System. The ratio between the target gene and β-actin gene band densities is used for quantitative evaluation.

Immunofluorescence with Confocal Microscopy For the immunofluorescence experiments, cells are fixed in 4% paraformaldehyde for 10 min and permeabilized with 0.2% Triton X-100 on ice for 10 min. Samples are blocked with 3% bovine serum albumin plus 0.02% Triton X-100 PBS for 30 min, incubated with antibody against NPM or Nup88 or Nup214 (at a working dilution of 1:100) separately overnight at 4 °C, then, FITC-labeled secondary antibody diluted in PBS is applied for 60 min. Hoechst 33258 (1 μg/ml) or PI (50 mg/l) is included in the penultimate wash step to visualize the DNA. Images are captured using a FV500 confocal microscope (Olympus, Tokyo, Japan).

Statistical Analysis Student’s t-test and one-way analysis of variance are used to determine the statistical significance of differences between values for various experimental and control groups. p values <0.05 was considered a significant difference.

RESULTS

Effects of Deguelin on Proliferation of Jurkat Cells The cytotoxicity of deguelin to Jurkat cells is calculated from the loss of cell viability using MTT assay. As shown in Fig. 1, treatment with various concentrations of 0, 5, 10, 20, 40, 60, 80, 160 nmol/l deguelin for 0, 12, 24, 48, 72 h result in a significant decrease in cell viability in a dose- and time-dependent manner in Jurkat cell line. The IC₅₀ value for 24 h is 46.03±0.13 nmol/l. With the exposure time increased, the IC₅₀ values decrease gradually.
Effects of Deguelin on Apoptosis of Jurkat Cells

To address the role of deguelin in conferring sensitivity to apoptosis, Jurkat cells are exposed to increasing concentration of deguelin, and then detected with DNA fragmentation assay and Annexin-V FITC/PI double-labeled cytometry. As a consequence, deguelin induces DNA fragmentation (Fig. 2A) and apoptotic cell death (Fig. 2B) in a dose-dependent manner (10—60 nmol/l). The degree of apoptotic cell death is quantified as the percentage of the Annexin-V FITC positive cells. There are little binding of Annexin-V FITC in untreated control. Magnification 1000×, scale bars=20 μm.

Fig. 2. Induction of Apoptosis by Deguelin in Jurkat Cells

Deguelin induces a dose-dependent apoptosis in Jurkat cells as determined by DNA fragmentation assay (A), Annexin-V FITC/PI double-labeled cytometry (B), and Hoechst 33258 staining assay (C). (A) Cells are incubated for 0—48 h with 40 nmol/l deguelin, or treated with various concentrations of 10—60 nmol/l for 24 h. (B) Cells are incubated with various concentrations of deguelin for 24 h and quantified of apoptotic cells, the diagram of curve corresponds to the graph of the Flow cytometry. (C) Cells are incubated for 24 h with 40 nmol/l deguelin, the arrows represent the apoptotic nuclear fragmentations. The figures are representative of three independent experiments. **p<0.01 with respect to untreated control.
becomes shrinkaged. While, the normal Jurkat cells present intact plasma membrane and order chromatin folding (Fig. 2C).

**Effects of Deguelin on the Regulation of NPM, Nup88 and Nup214 in Jurkat Cells** To study the mechanisms of anti-leukemia activity by deguelin, we first examine whether there exists constitutive deregulation of NPM protein in Jurkat cells. As a result, high protein level, as well as mRNA level of NPM appears in Jurkat cells compared with deguelin-treated cells (Figs. 3A, C). However, deguelin induces a dose-dependent decline of NPM both at transcriptional and translational level.

Since the important role of nucleoporins in the nucleocytoplasmic transport of macromolecules, we then study the expression of Nup88 and Nup214 in Jurkat cells. Same as above, deguelin also induces the declination of Nup88 and Nup214 at the mRNA level (Figs. 3A—C).

**Disposition of NPM, Nup88 and Nup214 in Jurkat Cells** To visualize the subcellular locations of NPM, Nup88 and Nup214 better, confocal microscope is used. We find NPM appears high fluorescence intensity in nucleus, especially in nucleoli, with an average optical density (OD) value being 220.02 ± 110.06. Besides, most of NPM protein shuttles to cytoplasm during cell mitosis. When cultured with 40 nmol/l deguelin, the fluorescence density drops to 130.86 ± 17.96 (Fig. 4A). On the other hand, Nup88 and Nup214 are found to be dispersed diffusely in the cytoplasm and nuclear envelope, with the mean fluorescence densities being 148.29 ± 27.40 and 161.80 ± 22.19 separately. However, deguelin induces a dramatic decline in fluorescence density to 54.70 ± 16.90 and 61.23 ± 14.52 correspondingly. Moreover, both nucleoporins are relocated to the nuclear envelope (Figs. 4B, C).

**DISCUSSION**

An emerging but still poorly explored link is that between nucleocytoplasmic transport and cell apoptosis, proliferation or differentiation, supporting the notion that nucleocytoplasmic transport of signal transducers and execution factors, may be a crucial and even essential aspect of cellular homeostasis. Mechanisms that regulate nucleocytoplasmic transport of proteins may provide novel opportunities for drug development.

Deguelin, a natural plant extract, isolated from plants in Mundulea sericea family, has been observed to inhibit tumor cell growth or induce cell apoptosis in several mammalian cell culture systems. However, the effect and mechanism of action of deguelin on T cell leukemia is not well known. In this study, we show that deguelin shows strong antiproliferative potency on Jurkat cells in a time- and dose-dependent manner, with IC_{50} for 24 h being (46.03 ± 0.13) nmol/l. Moreover, deguelin also presents potent apoptosis-induction activity with typical morphological changes associated with apoptosis. It suggests that deguelin is an effective anti-leukemia reagent through the ability of inducing growth-arrest and apoptosis.

Ample amount of evidence has implicated NPM deregulation in promoting tumor cell proliferation, differentiation and apoptosis. In haematological malignancies, NPM is downregulated in HL-60 cells during retinoic acid-induced differentiation and sodium butyrate-induced apoptosis. Also, decreased expression of NPM is involved in phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced differentiation of K562 cells. It is conceivable that high levels of NPM in malignant cells might support aberrant cell growth by sustaining the ribosome machinery, as well as promotion proliferation and inhibition of distinct pro-apoptotic
On the other hand, NPM1 is one of the most frequent targets of genetic alterations in hematological malignancies, especially in de novo AML. Therefore, the complex behavior of NPM protein demands further studies, including the molecular mechanisms and biological consequences of its altered expression and localization. In this study, we have shown that NPM prevails in the nucleus, especially in the nucleoli of the Jurkat cells. However, deguelin induces a dramatic downregulation of NPM at transcriptional level. What’s more, in deguelin-treated cells, NPM is found to be scattered more concentrated in the nucleoli with low fluorescence density. It is plausible to propose that NPM function as a proto-oncogene rather than an anti-oncogene in Jurkat cells.

Since the important role of nucleocytoplasmic transport of macromolecules in cellular homeostasis, we suggest the irregular transcription or subcellular localization of NPM, or other signal transducers and execution factors, might be the result of abnormal nucleocytoplasmic transport. It is reported recently that sumoylation of NPM regulated its subcellular localization and influences cell proliferation and its transcriptional level. What’s more, in deguelin-treated cells, NPM is found to be scattered more concentrated in the nucleoli with low fluorescence density. It is plausible to propose that NPM function as a proto-oncogene rather than an anti-oncogene in Jurkat cells.

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nucleocytoplasmic transport required to meet the increased proliferation rate and upregulated transcriptional activity of NPM in Jurkat cells. Deguelin, as a potent antileukemia agent, might rectify the deregulation of Nup88 and Nup214, thus influence the activities of cell proliferation and apoptosis.

Taken together, our results imply that deguelin also presents potent effects on growth-arrest and apoptosis-induction in Jurkat cells in vitro, which might be related to the regulation of nucleophosmin and some key nucleoporins, such as: Nup88 and Nup214. It may represent a new remedy for acute leukemia.

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