Location of NLS-RARα protein in NB4 cell and nude mice

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Abstract. In the majority of acute promyelocytic leukemia (APL) cases, translocons produce a promyelocytic leukemia protein-retinoic acid receptor α (PML-RARα) fusion gene. Studies have reported that neutrophil elastase (NE) cleaves bcr-1-derived PML-RARα in early myeloid cells, leaving only the nuclear localization signal (NLS) of PML attached to RARα. NLS-RARα promotes cell growth and inhibits differentiation in response to ATRA. However, the mechanisms by which NLS-RARα affects cell biological characteristics are yet to be fully elucidated. The present study found that the location of RARα was altered after it was cleaved by NE. Firstly, NE was overexpressed during the preparation of recombinant plasmid NB-4/pCMV6-NE-Myc to cleave PML-RARα. The total protein expression levels of myc and NE and expression levels of NLS-RARα in nucleoprotein were detected by western blotting. Location of NLS-RARα protein was detected by immunofluorescence and confocal laser scanning. Secondly, a nude mice model was constructed and NE protein, NLS-RARα and RARα protein assays, and the location of NLS-RARα and RARα proteins were assessed as described. The present results showed that, compared with the control groups, the location of NLS-RARα protein was predominantly detected in the nucleus, whereas RARα was mainly distributed in the cytoplasm. These findings were consistent with those of the nude mice model, and these may be used as a foundation to explain the occurrence mechanism of APL.

Introduction

As a member of the nuclear steroid/thyroid hormone receptor superfamily, retinoic acid receptor-alpha (RARα) is encoded by the RARα gene mapped on chromosome 17q21 in humans (1). RARα gene near the breakpoint of acute promyelocytic leukemia (APL) is thought to have a key role in the pathogenesis of APL, as RARα acts as a ligand-dependent transcription factor to modulate the expression of target genes after stimulated by ATRA (2-4). Some studies declared that after it is synthesized and has undergone appropriate modification and stimulation by ATRA, RARα can be transported from the cytoplasm to the nucleus. Thus, it is located both in cytoplasm and nucleus in a physiological manner. Promyelocytic leukemia (PML), which is encoded by a PML gene mapped on chromosome 15q22 in humans, contains a nuclear localization signal (NLS), an α-helical coiled-coil region and B-Boxes (5). In 1991, it was discovered that the consistent chromosomal translocation of APL, t (15;17), fused the RARα gene to the PML gene on chromosome 15 (6), yielding the fusion protein PML-RARα (7), which represents the etiologic agent of APL. This translocation has become the definitive marker for the disease and is detected in 95% of patients with APL (8). The aberrant PML-RARα fusion product has a vital role in APL (9). It produces PML-RARα protein to inhibit the transcription of myeloid differentiation-associated gene, resulting in the blocking of the differentiation of granulocytes at the promyelocytic stage, thus promylcytes were abnormally accumulated in the bone marrow (10). At last it leads to the occurrence of APL.

PML-RARα fusion protein does not always have a role as a whole; it has been demonstrated that PML-RARα fusion protein may be cut into two variant proteins in early myeloid cells by neutrophil elastase (NE), which is an early myeloid-specific serine protease (11). This type of cleavage action separates NLS from the PML gene and attaches it to RARα, which then named NLS-RARα (61 kDa). These data suggested that the disruption of RARα protein may be the critical cause of APL. Previous experimental studies in our laboratory have demonstrated that NLS-RARα protein interacts with JTV1 protein, ubiquilin 1 protein and glutamate ammonia ligase by using a yeast two-hybrid system and co-immunoprecipitation techniques (12,13). NLS-RARα is able to promote cell proliferation and induce cell differentiation, which is inhibited by ATRA though the expression of c-myc (14).

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As a specific signal mediated protein, NLS is a specialized sequence present on certain proteins. NLS, which can help NLS-containing cargo protein reach the nucleus through the nuclear pore, was identified by the corresponding nuclear transport protein (5).

In order to explore the influence of NLS on NLS-RARα protein location, pCMV6-NE-Myc plasmid was electroporated into NB4 cells and the subcutaneous tumor model in nude mice was constructed. Cells were detected and analyzed by western blotting, immunofluorescence and confocal laser scanning. The results of the present study may provide the foundation for further research of the mechanism of occurrence of APL.

Materials and methods

Cell line and culture. Human K562 cells (Institute for Biological Sciences, Shanghai, China) were maintained in RIPA 1640 culture medium supplemented with 10% fetal bovine serum (FBS; both Gibco; Thermo Fisher Scientific, Inc. Waltham, MA, USA). Human NB4 cells were stored in our own laboratory and maintained in RIPA 1640 culture medium supplemented with 10% fetal bovine serum. Cells were grown at 37˚C in an atmosphere containing 5% CO₂. All of cells were cultured in medium that was replaced every two days. A vector map is shown in Fig. 1.

Electroporation and select. pCMV6-NE-Myc and pCMV-KZ-Myc plasmids were recombined by our own laboratory. Prior to electroporation, NB4 cells were washed with RIPA 1640 culture medium, counted, resuspended in culture medium to a cell density of 1x10⁶ cells/ml, and incubated in an ice bath for 5 min. Cells and plasmids (20 µg/ml) were mixed in RIPA 1640 culture medium and transferred into an electroporation cuvette (Lonza Inc., Allendale, NJ, USA). The electroporation apparatus (Lonza Inc.) was set at 250 V for 10 msec using a 0.4-mm cuvette. Following incubation at 37˚C for 30 min, 1 ml fresh RIPA 1640 culture medium supplemented with 10% fetal bovine serum was added and the cells grew at 37˚C in an atmosphere containing 5% CO₂. Cells were selected by G418 (300 µg/ml; Invitrogen; Thermo Fisher Scientific, Inc.) pathogen-free environment with a 12-h light/dark cycle, and provided with food and water ad libitum. Tumors were housed in a temperature-controlled (24-25˚C and 50% humidity) pathogen-free environment with a 12-h light/dark cycle, and provided with food and water ad libitum. Tumors were stripped in the first 10 days, cut into pieces and dissociated into single cell suspensions by Trypsin (0.25%; Invitrogen; Thermo Fisher Scientific, Inc.), for >5 weeks. Western blotting was used to assess the myc expression. Cells which stably expressed myc protein were referred to as NE-NB4. In the negative control group, KZ-NB4 cells were processed in the same way.

Total protein, cytoplasmic and nuclear protein extraction. For total protein extraction, cells in each group were washed with ice-cold phosphate-buffered saline and lysed in RIPA 1640 solution containing a protease inhibitor cocktail. Cytoplasmic and nuclear protein extraction was subsequently performed. Cells in each group were washed with ice-cold phosphate-buffered saline and treated with a nuclear protein extraction kit (Beyotime Institute of Biotechnology, Haimen, China). Following concentration determination by the BCA method, proteins were stored at -80˚C.

Western blot assay. A total of 100 µg protein from each group was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. These membranes were blocked for 4 h at room temperature in 5% skim milk then incubated with the following primary antibodies overnight at 4˚C: Anti-myc monoclonal antibody (1:500; cat. no. 2276; Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit polyclonal antibody against RARα (1:500; cat. no. sc-366090) and rabbit polyclonal antibody against NE (1:500; cat. no. sc-9520; both Santa Cruz Biotechnology, Dallas, TX, USA). Following washing, the membranes were incubated with secondary goat anti-mouse antibody (cat. no. ZM-0491) or goat anti-rabbit antibodies (both 1:1,000; cat. no. ZA-0448; both Zhongshan Goldenbridge Biotechnology Co., Ltd., Beijing, China) for 1 h at 37˚C. Following washing three times (10 min each time) in TBST, immunoreactive complexes were visualized using an enhanced chemiluminescence system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). β-actin or Histone H3 served as an internal positive control. Protein bands were visualized using the Quantity One Software version 4.5.2 (Bio-Rad Laboratories, Inc.).

Immunofluorescence and confocal laser scanning. Following washing with PBS, the cells in each group were smeared onto slides, fixed with 4% paraformaldehyde for 20 min at 4˚C and permeabilized in 0.1% Triton at room temperature for 10 min. Prior to the primary antibody reaction, the cells were incubated with 10% goat serum (Beyotime Institute of Biotechnology) for 30 min at room temperature. Subsequently, the slides were incubated with rabbit polyclonal primary antibody against RARα (1:200; Santa Cruz Biotechnology, Inc.) at 4˚C for 8 h, followed by incubation with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (1:200; Zhongshan Goldenbridge Biotechnology Co., Ltd.) at room temperature for 1 h. Following counterstaining with 4'-diamino-2-phenylindole dihydrochloride (DAPI; 1 µg/ml; 5 min) or PI (propidium iodide; 1 µg/ml; 2 min), cells were visualized by microscopes.

Cells were counterstained with DAPI were visualized by fluorescence microscope (x400; Nikon Corp., Tokyo, Japan), and the cells counterstained with PI were scanned using confocal laser scanning microscope (magnification, 400x; Leica Microsystems GmbH, Wetzlar, Germany).

Nude mice models. A total of nine male nude mice (7-8 weeks old; 18-20 g) were randomly divided into three groups and inoculated by subcutaneous injection into the right front armpits with NE-NB4, NB4 and K562 cells, respectively. Animals were housed in a temperature-controlled (24-25˚C and 50% humidity) pathogen-free environment with a 12-h light/dark cycle, and provided with food and water ad libitum. Tumors were stripped in the first 10 days, cut into pieces and dissociated into single cell suspensions by Trypsin (0.25%; 3 min). These cells were respectively named as Tumor-NE-NB4, Tumor-NB4 and Tumor-K562, and were analyzed by western blot, immunofluorescence and confocal laser scanning analysis, as outlined. All animal experiments were performed according to national laws. This study was approved by the Ethics Committee of Chongqing Medical University (Yongchuan, China).

Statistical analysis. Data was presented as the mean ± standard deviation. Statistical analysis was performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Independent sample t-test was employed for compare the means between two groups. P<0.05 was considered to indicate a statistically significant difference.
Results

Expression of myc and NE in total protein of NE-NB4 cells.
Western blot assay results demonstrated the presence of myc protein was confirmed in NE-NB4 and KZ-NB4 cells, compared with the NB4 group (P<0.05; Fig. 2A). Western blot assay also showed that NE protein was expressed in NE-NB4 cells (P<0.05; Fig. 2B).

![Vector map](image1)

**Figure 1.** Vector map.

![Expression of myc and NE in NE-NB4 cells](image2)

**Figure 2.** Expression of myc and NE in NE-NB4 cells. (A) Protein expression levels of myc were detected by western blot assay. When compared with the NB4 group, myc expression in the NE-NB4 and KZ-NB4 cells increased significantly (P<0.05). (B) Protein expression of NE was assessed by western blot assay. Protein expression levels of NE in the NE-NB4 cells were significantly increased, as compared with the controls (P<0.05). Data were expressed as the mean ± standard deviation. *P<0.05. NE, neutrophil elastase.

![Expression of NLS-RARα in nucleoprotein of NE-NB4 cells](image3)

**Figure 3.** Expression of NLS-RARα in nucleoprotein of NE-NB4 cells. (A) Protein expression levels of NLS-RARα were detected in nucleoprotein of NE-NB4 cells by western blot assay. Protein expression levels of NLS-RARα in the NE-NB4 cells were significantly increased, as compared with KZ-NB4, NB4 and K562 cells. (B) Expression of PML-RARα in the cytoplasmic protein of NE-NB4 cells significantly decreased when compared with KZ-NB4 and NB4 cells (P<0.05). Data were expressed as the mean ± standard deviation. *P<0.05. NLS-RAR, nuclear localization signal-retinoic acid receptor; PML, promyelocytic leukemia protein; NE, neutrophil elastase.
Figure 4. RARα proteins in the (A-C) NE-NB4, (D-F) electrical KZ-NB4, (G-I) NB4 and (J-L) K562 groups were visualized by immunofluorescence staining with anti-RARα antibody. Target protein was stained green by FITC; the nucleus was stained blue by DAPI. RAR, retinoic acid receptor; NE, neutrophil elastase; DAPI, 4’6’-diamino-2-phenylindole dihydrochloride; FITC, fluorescein isothiocyanate.

Figure 5. RARα and NLS-RARα proteins in the (A-C) NE-NB4, (D-F) electrical KZ-NB4, (G-I) NB4 and (J-L) K562 groups were visualized by confocal laser scanning staining with anti-RARα antibody. Target protein was stained green by FITC; the nucleus was stained blue by PI. RAR, retinoic acid receptor; NE, neutrophil elastase; PI, propidium iodide; FITC, fluorescein isothiocyanate.
Expression of NLS-RARα in nucleoprotein of NE-NB4 cells. Western blot assay results showed that nucleoprotein of NE-NB4 cells confirmed the presence of NLS-RARα protein (P<0.05; Fig. 3A). Western blot assay also indicated that PML-RARα were expressed in the cytoplasmic protein of KZ-NB4 and NB4 cells (P<0.05; Fig. 3B).

Immunofluorescence detection in NE-NB4 cells. The FITC area and nuclear DAPI staining areas almost overlapped in NE-NB4, suggesting that NLS-RARα protein in the NE-NB4 group was predominantly presented in the nucleus (Fig. 4A-C). However, in the KZ-NB4 (Fig. 4D-F), NB4 (Fig. 4G-I) and K562 groups (Fig. 4J-L), the FITC-stained areas were markedly larger than the nuclear DAPI-stained areas. The nuclear areas were lightly stained, suggesting that other types of RARα proteins in the KZ-NB4, NB4 and K562 groups were presented in the cytoplasm and nucleus, and were predominantly distributed in the cytoplasm.

Confocal laser scanning of NE-NB4 cells. FITC-stained and nuclear PI-stained areas almost overlapped in NE-NB4, suggesting that NLS-RARα protein in the NE-NB4 group was predominantly presented in the nucleus (Fig. 5A-C). However, in the KZ-NB4 (Fig. 5D-F), NB4 (Fig. 5G-I) and K562 (Fig. 5J-L) groups, the FITC-stained areas were markedly larger than nuclear PI-stained areas. The nuclear areas were lightly stained, suggesting that other types of RARα proteins in the KZ-NB4 group, NB4 group and K562 group were localized to the cytoplasm and nucleus, and were predominantly distributed in the cytoplasm.
Expression of NE in total protein of tumor-NE-NB4 cells. Western blot assay results indicated that the NE protein was expressed in the Tumor-NE-NB4 cells, while NE protein expression was low in Tumor-NB4 and Tumor-K562 cells (P<0.05; Fig. 6).

Expression of NLS-RARα in nucleoprotein of Tumor-NE-NB4 cells. Western blot assay results demonstrated that the nucleoprotein of Tumor-NE-NB4 cells confirmed the presence of NLS-RARα protein (P<0.05; Fig. 7A). The results of western blot assay also showed that PML-RARα was significantly expressed in the cytoplasmic protein of Tumor-NB4 cells, as compared with Tumor-NB4 and Tumor-K562 cells in the present study (P<0.05; Fig. 7B).

Immunofluorescence detection in tumor cells. The FITC-stained and nuclear DAPI-stained areas almost overlapped in Tumor-NE-NB4 cells, which suggested that NLS-RARα protein in Tumor-NE-NB4 group was predominantly presented in the nucleus (Fig. 8A-C). However, in the electrical Tumor-NB4...
(Fig. 8D-F) and Tumor-K562 (Fig. 4G-I) groups, the FITC-stained areas were significantly larger than those of the nuclear DAPI-stained areas. These findings suggested that other types of RARα proteins in the Tumor-NB4 and Tumor-K562 groups were presented in the cytoplasm and nucleus.

Confocal laser scanning tumor cells. The FITC-stained and nuclear PI-stained areas almost overlapped in Tumor-NE-NB4 cells, suggesting that NLS-RARα protein in the Tumor-NE-NB4 group was predominantly presented in the nucleus (Fig. 9A-C). However, in the electrical Tumor-NB4 (Fig. 9D-F) and Tumor-K562 (Fig. 9G-I) groups, FITC-stained areas were significantly larger than those of the nuclear PI-stained areas, suggesting that other types of RARα proteins in the Tumor-NB4 and Tumor-K562 groups were in the cytoplasm and nucleus.

Discussion

The PML-RARα gene has an important role in the initiation and progression of APL and is a marker of the disease. The PML-RARα protein blocks the differentiation of hematopoietic progenitor cells [15]. The importance of the PML-RARα gene has been confirmed in transgenic mice [16]. Direct DNA-binding is indispensable for PML/RARA to transform hematopoietic cells and the disruption of PML-nuclear bodies is not sufficient for full cell leukemic transformation [17]; however, disruption of PML may facilitate the acquisition of leukemia-promoting mutations by disabling oncogene-induced senescence [18].

However, the entire PML-RARα fusion protein is not necessary for the development of APL [19,20]. Lane and Ley [11,21] found that the PML-RARα protein can be cleaved by NE in early myeloid cells, resulting in two new mutants: PML, named NLS, and RARα, named NLS-RARα. RARα and PML are regulatory proteins implicated in various aspects of differentiation and development [22] and apoptosis and cellular senescence [23,24], respectively. Mutations of RARα and PML may cause aberrant subcellular localization, insufficient sumoylation, and/or multimerization [25]. It has previously been demonstrated that fusion of RARα with self-associating domains is sufficient to render RARα leukemogenic [26], and the combination of staurosporine and ATRA may overcome granulocytic differentiation block in retinoid-resistant APL cell lines [27].

Our previous study indicated that PML (NLS-) is a putative tumor promoter factor in human leukemia cells, and downregulation PML (NLS-) expression inhibited the proliferation of HL-60 cells and induced apoptosis [28]. Wild-type RARα inhibits cell growth and promotes differentiation in response to ATRA, whereas NLS-RARα has the opposite effect [15]. Using a yeast two-hybrid system and co-immunoprecipitation techniques, it was demonstrated that NLS-RARα protein interacted with JTV1 protein, ubiquitin 1 protein and glutamate ammonia ligase (12-14). NLS-RARα was able to promote cell proliferation and induce cell differentiation, which is inhibited by ATRA though the expression of c-myc (12-14). In response to ATRA, wild-type RARα inhibits cell growth and promotes differentiation, whereas NLS-RARα does the opposite. We hypothesize that this difference may be associated with abnormal cellular localization of the RARα domain, which may result in abnormal protein-protein interactions. This would alter the original signal pathway and biological function, and subsequently promote the occurrence and development of APL.

The NB4 cell line is widely used in the investigation of APL, because it exhibits the typical t (15; 17) (15q22; 17q21) translocation and the characteristics of PML-RARα fusion gene [9-11,21]. However, it was discovered that there are no NE enzyme in NB4 cells [10]. Therefore, the present study established a NE-NB4 model by electroproporation to explore the location of NLS-RARα. The results showed that NLS-RARα may be detected in the nucleus by western blotting, and the results of immunofluorescence and confocal laser scanning suggested that it was predominantly located in the cell nucleus. This would alter the original signal pathway and biological function, thus promoting the occurrence and development of APL.

In conclusion, the abnormal cellular localization of NLS-RARα protein, which is likely to result in abnormal protein-protein interactions, may be associated with cell growth and differentiation and this may be one of the occurrence mechanisms of APL.

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