c-Jun N-terminal kinase mediates AML1-ETO protein-induced connexin-43 expression

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Received 28 February 2007
Available online 8 March 2007

Abstract

AML1-ETO fusion protein, a product of leukemia-related chromosomal translocation t(8;21), was reported to upregulate expression of connexin-43 (Cx43), a member of gap junction-constituted connexin family. However, its mechanism(s) remains unclear. By bioinformatic analysis, here we showed that there are two putative AML1-binding consensus sequences followed by two activated protein (AP)1 sites in the 5'-flanking region upstream to Cx43 gene. AML1-ETO could directly bind to these two AML1-binding sites in electrophoretic mobility shift assay, but luciferase reporter assay revealed that the AML1 binding sites were not indispensable for Cx43 induction by AML1-ETO protein. Conversely, AP1 sites exerted an important role in this event. In agreement, AML1-ETO overexpression in leukemic U937 cells activated c-Jun N-terminal kinase (JNK), while its specific inhibitor SP600125 effectively abrogated AML1-ETO-induced Cx43 expression, indicating that JNK signaling pathway contributes to AML1-ETO induced Cx43 expression. These results would shed new insights for understanding mechanisms of AML1-ETO-associated leukemogenesis.

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Keywords: AML1-ETO; Connexin-43; Leukemia; c-Jun N-terminal kinase (JNK)

Gap junctional intercellular communication (GJIC), a specialized channel assembled from connexins (Cx) family members, serves a common purpose of allowing direct transfer of small metabolites, second messengers, and electrical signals from the cytoplasm of one cell to that of the adjacent cells [1]. Among Cx family, Cx43 is a frequently investigated and most universal member found in human. In addition to contributing to GJIC, increasing evidence supported that Cx43 also inhibits cell proliferation in gap junction-dependent and independent mechanisms, and contributes to pathogenesis of many diseases [2,3]. For instance, Cx43 is deregulated in cancers of various origins such as endometrial cancer, ovarian cancer and lung cancer [4,5]. The restoration of Cx43 expression by transfection leads to growth suppression and/or differentiation/apoptosis induction of cancer cells [6]. Additionally, Cx43 overexpression also regulates various angiogenesis-linked proteins and inhibits the malignant properties of breast cancer cells via GJIC-independent regulation of epithelial to mesenchymal transition and angiogenesis [7]. Based on these important findings, Cx43 is regarded as a hypothetical tumor suppressor and possibly acts as a potential anti-oncogenic target for chemoprevention and/or chemotherapy [8].

The chromosomal translocation t(8;21) is associated with 10–15% of de novo acute myeloid leukemia (AML) cases and up to 40% of M2-type AML cases of the French–American–British classification. The translocation generates AML1-ETO fusion protein, which consists of 177 N-terminal amino acid residues of AML1 and nearly the entire ETO (eight-twenty one) protein [9]. As widely accepted, the AML1-ETO gene inhibits the differentiation of leukemic cells towards granulocytic, monocytic or erythroid cells [9]. However, inducible AML1-ETO expression also induces growth arrest and makes leukemic cells prone to both extrinsic and intrinsic apoptosis in leukemic cell line [3,10]. More recently, we reported that AML1-ETO expression upregulates the transcription of Cx43 gene in
leukemic U937 cells, which plays a role in AML1-ETO-induced growth arrest [3]. Herein, we investigated the molecular mechanisms by which AML1-ETO upregulates Cx43 expression. Our results showed that AML1-ETO bound to two consensus DNA binding sequences for AML1 in the regulatory region of Cx43 gene, but they were not indispensable for Cx43 induction by AML1-ETO. On the contrary, AML1-ETO-activated c-Jun N-terminal Kinase (JNK) signaling pathway is highly involved in this process.

Materials and methods

Cell lines and reagents. Human leukemic cell lines used in this study included human acute monocytic leukemia U937 cell line and U937-A/E 9/14 cells with conditional AML1-ETO expression that was generated by the edysone inducible system from parental U937 cells as described previously [11]. For induction of AML1-ETO expression in U937-A/E 9/14 cells, 5 μM ponasterone A (Invitrogen, Groningen, NL) was added to the medium, and Western blots were performed to confirm AML1-ETO expression. Leukemic cell lines and the non-hematopoietic cell lines 293 and Hela were, respectively, cultured in RPMI 1640 and DMEM medium (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (FCS, Hyclone, Logan, UT) in a humidified incubator at 37 °C and 5% CO2/95% air. SP600125 was purchased from Calbiochem Inc. (San Diego, CA).

Plasmids and transfection. The pCMV5-AML-1B, pCMV5-ETO, pCMV5-AML1-ETO and pCMV5-CBFβ plasmids were generous gifts from Dr. Scott W. Hiebert [12]. For EMSA and luciferase assays, the coding regions of full-length AML-1B, ETO, and AML1-ETO cDNAs were amplified by PCR from the parental plasmids pCMV5-AML-1B, pCMV5-ETO, pCMV5-AML1-ETO, respectively, and PCR products were then cloned into the pFLAG-CMV4 vector (Sigma, St. Louis, MO), in equal loading of protein.

Electrophoretic mobility shift assay (EMSA). 293 cells were transfected with pFLAG-AML1-ETO or pFLAG-AML1B for 48 h, and then nuclear extracts were prepared using the Nuclear and Cytoplasmic Extraction Kit from Dr. Scott W. Hiebert [12]. For EMSA and luciferase assays, the coding regions of full-length AML-1B, ETO, and AML1-ETO cDNAs were amplified by PCR from the parental plasmids pCMV5-AML-1B, pCMV5-ETO, pCMV5-AML1-ETO, respectively, and PCR products were then cloned into the pFLAG-CMV4 vector (Sigma, St. Louis, MI), in frame with the FLAG coding cDNA. The fidelity of all PCR products was verified by DNA sequencing (Invitrogen, Shanghai, China). Plasmids were transfected into 293 or Hela cells using the Polyfect transfection reagent (Qiagen, Valencia, CA).

Western blots. Protein extracts were equally loaded on 10–12% SDS-polyacrylamide gel and electrophoretically transferred to NC membranes (Schleicher & Schuell, Dassel, Germany). The membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20, and then incubated with each primary antibody overnight at 4 °C. The following primary antibodies were used in this study: Cx43 (C9093, Sigma–Aldrich, St. Louis, MO), ETO (sc-9737, Santa Cruz Biotechnology, Santa Cruz, CA), AML1 (sc-8563, Santa Cruz Biotech, Santa Cruz, CA), FLAG (F1804, Sigma–Aldrich, St. Louis, MI), rabbit polyclonal antibodies for P38, ERK, c-Jun, JNK and their phosphorylated forms as well as phospho-PKCα (Ser643) (Cell Signaling, Beverly, MA), rabbit polyclonal anti-PKCδ antibody (sc-937, Santa Cruz Biotech). Finally, blots were incubated with HRP-linked secondary antibody (Cell Signaling). Detection was performed by chemiluminescence phototope-HRP kit according to the manufacturer’s instructions (Cell Signaling). Blots were also re-probed with anti-β-actin (Merck, Darmstadt, Germany) antibody to ascertain equal loading of protein.

Statistical analysis. The Student’s-test was used to compare the difference between two different groups. A value of p < 0.05 was considered to be statistically significant.

Results

AML1B and AML1-ETO bind to the Cx43 promoter

AML1-ETO protein contains DNA-binding Runt domain at N-terminus of wild type AML1 protein which can bind to AML1 binding consensus motif (TGT/CGGT) of promoters of AML1-targeted genes [13]. To understand the mechanisms by which AML1-ETO increases Cx43 expression, we first analyzed 2 kb sequence of 5′-flanking region upstream to the transcription starting site of Cx43 gene. One typical (TGT/CGGT) and one complementary (ACCACA) AML1 binding sites could be seen, which were respectively located at −1385 to −1380 bp and −1069 to −1064 bp of Cx43 gene (Fig. 1A). Thus, we determined if AML1 or AML1-ETO protein in fact binds to these two putative AML1 binding sites. For this purpose, FLAG-AML1B or FLAG-AML1-ETO cDNA was transfected into 293 cells, and their nuclei were extracted. Western blots confirmed higher expression of nuclear FLAG-AML1B or FLAG-AML1-ETO protein (Fig. 1C). With
these nuclear extracts and two putative DNA-binding oligonucleotides as probes, EMSAs were performed. When nuclear extract with FLAG-AML1B (left, Fig. 1D) or FLAG-AML1-ETO overexpression (right, Fig. 1D) was incubated with the biotin-labeled oligonucleotide, a specific gel shift complex (lanes 2, 6, 10, 14, Fig. 1D) could be seen, which was greatly competed by the addition of 200-fold molar excess of unlabeled self-competitor, especially in the presence of AML1-ETO (lanes 3, 7, 11, 15, Fig. 1D). Furthermore, a supershifted band was recognized with anti-FLAG antibody (lanes 4, 8, 12, 16, Fig. 1D), supporting the specificity of the interaction between AML1B or AML1-ETO and these two oligonucleotides. According to this test, we concluded that both AML1-ETO and AML1B specifically bind to two putative AML1-binding sites of the promoter of Cx43 gene.

AML1-ETO activates the Cx43 promoter

In addition to AML1-binding sites, two activator protein 1 (AP1) sites (TGAG/CTCA) were also found at −44 to −38 bp and −1000 to −994 bp of Cx43 promoter (Fig. 1A). To figure out whether binding of AML1 or AML1-ETO impacts transcription driven by these motifs of Cx43 promoter, three fragments of Cx43 promoter containing AML1 and/or AP1 binding sites were subcloned into Luc reporter plasmid pGL3-basic to form AML1/AP1-Luc, AML1-Luc and AP1-Luc, respectively. Expression of FLAG-AML1-ETO and FLAG-AML1B were confirmed by Western blots. AML1-binding oligonucleotide probes (−1403 to −1364 bp and −1086 to −1047 bp) from Cx43 promoter were incubated with nuclear extracts from pFLAG-AML1B or pFLAG-AML1-ETO transfected 293 cells with or without excess (200-fold molar) of unlabeled competitor self-probe. Shaded triangle points to AML1 or AMl1-ETO-probe complex. Specific supershift of the band-shift complex pointed by empty triangle was produced with anti-FLAG antibody.

AML1-ETO induces phosphorylation of JNK in leukemic cell line

AP1 is a heterodimer nuclear protein composed of the c-Jun and c-Fos. This dimer binds to AP1 target sequences, while JNK activates c-Jun gene expression.
through binding to its AP1 site [15]. Hence, we tested whether AML1-ETO induction activated JNK and two other members (P38 and ERK) of mitogen-activated protein (MAP) kinase family, as expressed by their phosphorylated levels. As for this, U937-A/E 9/14/18 and U937 cells were incubated with 5 μM ponasterone A for various hours, and total and phosphorylated JNK, P38 and ERK as well as PKCδ were detected by Western blots (Fig. 3).

As described [10,11], U937-A/E 9/14/18 subclone was generated by introducing ecdysone-inducible two vector system to conditionally express the AML1-ETO protein into human leukemic U937 cells. When this subclone

Fig. 2. AML1-ETO activates the Cx43 promoter. (A) AML1/AP1-Luc reporter plasmid was cotransfected with pCMV4, pCMV4-AML1B, pCMV4-ETO or pCMV4-AML1-ETO together with pCMV4-CBFβ and β-gal expression vectors into Hela cells. (B) pCMV4-AML1-ETO or its empty vector was cotransfected with AML1/AP1-Luc, AML1-Luc or AP1-Luc reporter plasmids, together with β-gal. Luc activity was measured and normalized by β-gal intensity. The values represent the mean with bar as SD of three independent experiments. Symbol (*) indicates p values compared with CMV4-transfected cells (A) and compared with AML1-Luc transfected cells in the presence of AML1-ETO (B).

Fig. 3. AML1-ETO induces phosphorylation of JNK in leukemic cell line. U937-A/E 9/14/18 cells and wild type U937 cells were incubated with 5 μM ponasterone A for the indicated hours. JNK, P38, ERK, PKCδ and their phosphorylated proteins, AML1-ETO and Cx43 were analyzed by Western blots. All experiments were repeated three times with similar results.
was treated with ponasterone A, AML1-ETO protein was time-dependently induced (Fig. 3). As reported previously [3], AML1-ETO induction caused the delayed increase of Cx43 protein in the engineered U937-A/E 9/14/18 cell line but not in its wild type U937 cells (Fig. 3). Consistent with a previous report [16], AML1-ETO induction increased phosphorylation of JNK protein in U937-A/E 9/14/18 but not in U937 cells, which began appeared at 18 h and became significant at 48 h after ponasterone A treatment (Fig. 3). Accordingly, AML1-ETO induction also increased activated expression of JNK-targeted protein c-Jun (Fig. 4A). Of note, AML1-ETO induction failed to affect levels of total and phosphorylated P38 and ERK as well as PKCδ proteins.

**Discussion**

The AML1 protein, which recognizes the consensus DNA-binding motif, has been shown to be able to regulate a number of hematopoietic differentiation-related genes, as reviewed by Licht J [18]. In most cases, AML1-ETO exerts the dominant negative effect on transcriptional activation of AML1-targeted genes [19], which is attributed to interaction of its ETO portion with nuclear co-repressors such as N-CoR and Sin3A that recruit the histone deacetylases, and lead to a lower level of histone acetylation and less-accessible chromatin [12,19]. However, the myeloid-specific promoter for the macrophage colony-stimulating factor (M-CSF) receptor is also synergistically up-regulated by AML1-ETO and AML1 proteins [20]. In addition, AML1-ETO also activates transcription of the anti-apoptotic BCL-2 gene, which requires the presence of an intact AML1-binding site in the 5′-flanking region of the BCL-2 gene, but AML1 does not modulate the activity of the BCL-2 promoter [21]. All these data indicate that AML1-ETO presents a complicated transcription-regulated activity. Previously, we showed that AML1-ETO fusion protein upregulates transcription of Cx43 gene in leukemic U937 cell line [3]. To understand mechanisms underlying this event, we firstly decided the presence of AML1-binding site(s) in the 5′-flanking region of Cx43 gene. Indeed, two putative AML1-binding sites could be found, although they were beyond 1kb of the 5′-flanking region from transcription starting site of Cx43 gene. These two sequences could bind to AML1 or AML1-ETO protein in EMSA experiments. However, these two AML1-binding sites-driven luciferase reporter failed to be modulated by both
AML1 or AML1-ETO expression, suggesting that DNA binding of AML1 or AML1-ETO protein on its own is insufficient to exert full activation of gene transcription. This is consistent with the view that AML1 seems to be a relatively weak activator of transcription [14], and AML1 is ineffective as an activator in the absence of cooperating factors bound to adjacent promoter sites, such as PU.1, CCAAT/enhancer-binding protein-α, and protein-binding sites [18]. By the way, the fact that increased Cx43 expression appeared far after AML1-ETO induction also supports that an indirect mechanism is involved.

Like other members of gap-junction-forming connexins, Cx43 exhibits a complex life cycle that is regulated at various levels. Many signaling pathways involve in the regulation of Cx43 expression, such as NF-κB and PI3 kinase pathways and MAP kinase signaling pathways [22,23]. Increased API1 synthesis can upregulate Cx43 transcription by estrogen [24]. Because AML1-ETO but not AML1 driven transcription of luciferase promoted by API1-binding sites in Cx43 promoter, we tested the hypothesis that Cx43 induction expression by AML1-ETO is mediated indirectly through API1 dimer. We showed that AML1-ETO induction in leukemic cell line activated JNK and increased c-Jun expression, as previous reported [16]. However, AML1-ETO failed to activate P38 and ERK. Mechanisms by which AML1-ETO activates JNK remained to be investigated. Because PKCδ was shown to activate JNK [25], we also tested whether PKCδ involves in AML1-ETO induced JNK activation. Our data rule this possibility out because AML1-ETO induction did not activate this kinase. As expected, the specific JNK Inhibitor SP600125 could block AML1-ETO induced up-regulation of Cx43, supporting that AML1-ETO induces Cx43 expression in a JNK-dependent mechanisms.

Although AML1-ETO was regarded as leukemogenic protein, various mouse models, including transgenic models, inducible systems and bone marrow transplant strategies, failed to reliably produce overt leukemia in the mice. This fact strongly suggests that additional mutagenic “hits” are required for AML1-ETO-related leukemogenesis [26]. Obviously, AML1-ETO-induced growth arrest would not be favorable for the propagation of cells harboring the t(8;21) translocation [3]. In this sense, it is rational to predict that genes contributing AML1-ETO-induced growth arrest became candidate(s) of additional mutagenic “hits” related with AML1-ETO-associated leukemia. Our previous work showed that down-regulation of Cx43 by siRNA partially restores the cell growth rates in the presence of AML1-ETO expression, suggesting that the upregulated Cx43 expression exerted a role in AML1-ETO-induced growth arrest [3]. Here, we showed that JNK signaling pathway mediates AML1-ETO-induced Cx43 expression. Therefore, whether JNK-related gene(s) possesses mutation and/or epigenetic alterations in AML1-ETO positive AML cells deserves consideration in future work. In conclusion, we demonstrated that JNK signal pathway mediates AML1-ETO-induced expression of Cx43. These results would shed new insights for exploring additional mutagenic “hits” for AML1-ETO-associated leukemia.

Acknowledgments

We thank Dr. Scott W. Hiebert for generously providing the plasmids and Dr. Michael Lübbert for kindly providing U937-A/E 9/14/18 cell lines. This work was supported in part by National Key Program (973) for Basic Research of China (NO2002CB512805), National Natural Science Foundation of China (30500217) and grants from Science and Technology Committee of Shanghai (04DZ14901).

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