Regulation of annexin II by cytokine-initiated signaling pathways and E2A-HLF oncoprotein

Takayuki Matsunaga, Toshiya Inaba, Hirotaka Matsui, Mayuko Okuya, Atsushi Miyajima, Takeshi Inukai, Tetsunori Funabiki, Mikiya Endo, A. Thomas Look, and Hidemitsu Kurosawa

In pro-B cell acute lymphoblastic leukemia (ALL), expression of the E2A-HLF fusion gene as a result of t(17;19)(p22;q13) is associated with poor prognosis, hypercalcemia, and hemorrhagic complications. We previously reported that the E2A-HLF fusion protein protects interleukin-3 (IL-3)–dependent lymphoid cells from apoptosis caused by cytokine starvation. Here, we report that annexin II, a surface phospholipid-binding protein and one of the proposed causes of the hemorrhagic complications of acute promyelocytic leukemia (APL), is also implicated in t(17;19)+ ALL. Annexin II was expressed at high levels in APL cells and in each of 4 t(17;19)+ leukemia cell lines, and annexin II expression was induced by enforced expression of E2A-HLF in leukemia cells. In IL-3–dependent cells, we found that annexin II expression was regulated by IL-3 mainly by Ras pathways, including Ras/phosphatidylinositol 3-kinase pathways. Moreover, E2A-HLF increased annexin II expression in IL-3–dependent cells in the absence of the cytokine. These findings indicate that E2A-HLF induces annexin II by substituting for cytokines that activate downstream pathways of Ras.

© 2004 by The American Society of Hematology

From the Division of Hematology, Department of Pediatrics, Dokkyo University School of Medicine, Tochigi, Japan; Department of Molecular Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Japan; Institute of Molecular and Cellular Bioscience, University of Tokyo, Japan; Department of Pediatrics, School of Medicine, University of Yamanashi, Japan; Department of Pediatrics, Yokohama City University, Japan; Department of Pediatrics, Iwate Medical University, Japan; and Pediatric Oncology Department, Dana-Farber Cancer Institute, Boston, MA.


Supported by Grants-in-Aid for Scientific Research (C), Japan Society for the Promotion of Science.

An Inside Blood analysis of this article appears in the front of this issue.

Reprints: Hidemitsu Kurosawa, Division of Hematology, Department of Pediatrics, Dokkyo University School of Medicine, Mibu, Tochigi 321-0293, Japan; e-mail: hidekuro@dokkyomed.ac.jp.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked “advertisement” in accordance with 18 U.S.C. section 1734.

© 2004 by The American Society of Hematology
Leukemia patients with the E2A-HLF fusion product and a tendency to bleed have laboratory data similar to those of patients with t(15;17)+ acute promyelocytic leukemia (APL). Major determinants for the pathogenesis of APL-associated coagulopathy have been investigated for the past decade, and factors expressed in leukemia cells that affect procoagulant or fibrinolytic activities have been identified (for a review, see Falanga et al21). These factors include tissue factor, inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), and members of the annexin family.22,23 One of these, annexin II, is reported to be a cause of the coagulopathy associated with APL.24 Annexin II is a 36-kDa protein that forms a complex with the annexin II light chain, a member of the S100 family. Annexin II is a calcium-regulated, phospholipid-binding protein expressed on the surfaces of endothelial cells, macrophages, and some tumor cells; it has been implicated in cell-cell adhesion and in plasminogen activation, and it may function as a cell surface receptor (for reviews, see Hajjar et al25,26). Surface localization of annexin II is absolutely dependent on micromolar-free Ca2+; the protein is stripped from the cell surface by EGTA (ethyleneglycotetraacetic acid).27 Annexin II is translocated to the endothelial cell surface within 16 hours of biosynthesis, and cell surface annexin II comprises approximately 4% of the total pool of annexin II in endothelial cells.27 The presence of the t(15;17) translocation is correlated with overexpression of annexin II, and annexin II mRNA expression is known to be down-regulated by treatment with all-trans retinoic acid (ATRA). Thus, the anomalous expression of annexin II on the surfaces of circulating APL cells may result in primary (annexin II dependent) hypercalcemia. However, surface expression levels of annexin II among leukemia cells with t(17;19) varied in a manner that suggests annexin II plays a role in the hypercalcemia or in the leukemic invasion rather than in the coagulopathy associated with this type of APL.

### Materials and methods

#### Cell culture and cell survival assay

Murine IL-3–dependent FL5.12 and Baf-3 pro-B lymphoid cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 0.3% 10T1/2-conditioned medium as a source of IL-3. Cell density was maintained below 10⁶/mL to avoid IL-3–independent growth. Establishment of FL5.12 cells expressing zinc-inducible E2A-HLF (FL5.12/E2A-HLF) or annexin II (FL5.12/annexin II) using the pMT-CB6+ eukaryotic expression vector (a gift from Dr F. Rauscher III, Wistar Institute, Philadelphia, PA) has been described previously.31 Stable transfectants of a truncated form of the human GM-CSF (hGM-CSF) receptor (β544 cells) and Ras mutants were described previously.14,28 Transfectants were maintained in medium containing either 0.6 μg/mL G418 or 200 μg/mL hygromycin. Human ALL cell lines that express E2A-HLF (UOC-B1, HAL-O1, YCUB-2, and Endo-kun) and other leukemia cell lines (Sup-B2, RS4;11, REH, 697, Jurkat, and NB-4) were cultured in RPMI 1640 medium containing 10% FCS; 697/E2A-HLF and 697/pMT cells that were transfected with the pMT/E2A-HLF vector or the empty pMT-CB6+ vector were described previously.29 For cell survival assays, annexin II or E2A-HLF expression was induced in FL5.12 cells by adding 100 μM ZnCl₂ for 16 hours before growth factor deprivation. IL-3 was removed by repeated centrifugation in fresh media, the cells were adjusted to 5 × 10⁷/mL on day 0, and culture continued without IL-3. Viable cell numbers were determined by trypan blue dye exclusion. Wortmannin and LY294002 were purchased from Sigma-Aldrich (St Louis, MO).

#### Cloning of annexin II full-length cDNA and Northern blot analysis

The annexin II cDNA was cloned from UOC-B1 cells by reverse transcription–polymerase chain reaction (RT-PCR) using upstream and downstream primers (5′-TCTCAGCTCTCGGCAGCGG-3′ and 5′-TTTCTAGACCTTTAGCT-3′). RT-PCR was performed with a cDNA Cycle Kit (Invitrogen, Carlsbad, CA). DNA sequencing confirmed that the insert sequence was identical to that of the annexin II cDNA.29 Total cellular or poly(A)–selected RNA was isolated using RNasy kits (Qiagen, Hilden, Germany) or Fast Track kits (Invitrogen), respectively, according to the manufacturers’ instructions. One microgram of messenger RNA or 20 μg total RNA was separated by electrophoresis in 1% agarose gels.
that lack E2A-HLF expression, annexin II protein was less abundant in these cells than in cells expressing E2A-HLF (lanes 6-9). The E2A-HLF fusion protein from each of the t(17;19)+ pro-B ALL cell lines migrated differently because the joining region at the fusion junction contains different numbers of inserted nucleotides, as described previously (fourth panel).1,2

Next, we tested whether E2A-HLF induces the expression of annexin II. For these experiments, 697 cells were transfected with a pMT-CB6+/E2A-HLF construct (see “Materials and methods”) to generate clones that express zinc-inducible E2A-HLF. Ectopic expression of E2A-HLF in 697 cells induced annexin II (Figure 2). However, 697/E2A-HLF cells grown without zinc showed a low (baseline) level of annexin II expression that was not observed in 697/pMT cells, possibly because of the leaky expression of E2A-HLF (Figure 2, lanes 1 and 7).

In control 697/pMT cells, which contain the empty vector, annexin II expression levels were unaffected by zinc, confirming that the observed changes in gene expression were induced by E2A-HLF, not by zinc (Figure 2, lanes 7-8). A comparison of the magnitude of induction of annexin II mRNA and protein suggested that E2A-HLF–induced annexin II not only by affecting mRNA levels but also through posttranscriptional mechanisms.

Annexin II is regulated by IL-3–initiated signaling pathways

We previously reported that E2A-HLF reverses apoptosis caused by cytokine starvation in murine IL-3–dependent lymphoid cells, such as Baf-3 and FL5.12 cells, indicating that the chimeric transcriptional factor at least partially substitutes for cytokine-initiated signaling.11 These findings prompted us to test whether expression of annexin II is under the control of cytokines. A marked decline in annexin II mRNA levels was observed within 8 hours or 4 hours of IL-3 deprivation in Baf-3 or FL5.12 cells, respectively (Figure 3). Similarly, annexin II protein expression declined, as shown by immunoblot analysis (Figure 3A-B, each third panel).

To identify the signaling pathways regulating the expression of annexin II, we used Baf-3 cells expressing a truncated form (residues 1-544) of the human βc chain (β544 cells).14 In β544 cells, stimulation with hGM-CSF activates pathways emanating from the βc chain proximal region, such as JAK2/STAT5, but it does not activate signaling pathways from the βc chain distal region, including Ras pathways.14 As expected, Bcl-xL mRNA, which is known to be regulated by JAK/STAT pathways,13,15

Results

Annexin II is induced by E2A-HLF

We performed Northern blot and immunoblot analyses to test the expression of annexin II in human leukemia cell lines. Because t(17;19)+ ALLs constitute only approximately 1% of childhood B-precursor ALLs,3 we used cell lines instead of primary patient samples. Four cell lines harboring the E2A-HLF chimeric protein (UOC-B1, HAL-O1, YCUB-2, and Endo-kun) uniformly expressed annexin II mRNA and protein at higher levels than NB-4, a t(15;17)-positive APL cell line used as a positive control (Figure 1, lanes 1-4, 10).

Sup-B2, a non-t(17;19) pro-B ALL cell line, lacked expression of annexin II (lane 5). Although annexin II mRNA was detected in other lymphoid leukemia cell lines (RS4;11, REH, 697, and Jurkat) containing 2.2 M formaldehyde, transferred to nylon membranes, and hybridized with an appropriate probe according to standard procedures.

Immunoblot analysis

Cells were solubilized in Nonidet P-40 lysis buffer (150 mM NaCl, 1.0% Nonidet P-40, 50 mM Tris, pH 8.0), and total cellular proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). After wet electrotransfer onto nitrocellulose membranes, the proteins were detected using appropriate antibodies and following standard procedures. Blots were then stained with primary antibodies followed by horseradish peroxidase–conjugated antirabbit immunoglobulin secondary antibodies and were subjected to chemiluminescent detection according to the manufacturer’s instructions (Amersham Pharmacia Biotech, Buckinghamshire, England). Anti–annexin II polyclonal antibody was purchased from BD Transduction Laboratories (Lexington, KY). Anti-HLF(C) antibody is described previously.27 For immunoblot analysis of cell surface eluates, cells (1 × 10^8) were washed 3 times with either Hanks balanced salt solution (HBSS; Invitrogen) alone or HBSS containing 4 mM CaCl2 (HBSS/Ca) and were treated with either HBSS alone or HBSS/Ca for 30 minutes on ice. Elutes were collected and were solubilized in Laemmli lysis buffer (10% glycerol, 2% SDS, 50 mM Tris, pH 8.0) and were separated using SDS-PAGE. Assays for eluted lactate dehydrogenase were performed as previously described.27

For personal use only.on July 15, 2017. By guest.
returned rapidly to its original level after the addition of hGM-CSF (Figure 4A, bottom panel). In contrast, annexin II mRNA was barely detectable 8 hours after IL-3 deprivation (Figure 4A, upper panel, lane 2) and remained low after the addition of hGM-CSF (lanes 3-10). These results suggest that signals originating from the βc chain proximal region are not important for the stable expression of annexin II but that signals from the βc chain distal region are indispensable for annexin II gene expression.

Oncogenic RAS and E2A-HLF induce annexin II expression in mouse pro-B lymphocytes

To further identify the pathways regulating annexin II gene expression, we used Baf-3 cells with dexamethasone (Dex)–inducible expression of a constitutively active form of Ras (RasG12V). The level of annexin II mRNA declined by 4 hours after IL-3 deprivation and Dex treatment, but it was restored after 24 hours (Figure 4B). These effects appeared to be induced by RasG12V, not by Dex itself, because annexin II mRNA was not induced in wild-type Baf-3 cells after the addition of Dex (data not shown). Induction of annexin II by RasG12V was partially reversed by the PI3-K inhibitor wortmannin (right panel), suggesting that PI3-K pathways are important for annexin II expression.

Indeed, the RasG12V/V45E mutant, which activates PI3-K but not Raf-MAPK pathways, induced annexin II at levels similar to those induced by RasG12V, and this effect was almost completely reversed by wortmannin (Figure 4C). These results were confirmed by the use of another PI3-K inhibitor, LY294002 (data not shown), suggesting that Ras/PI3-K is an important pathway in the regulation of annexin II gene expression under the control of cytokines.

Next, we tested whether enforced expression of the E2A-HLF chimera can induce the expression of annexin II in FL5.12 cells in the absence of IL-3. For these experiments, we established FL5.12 cells expressing zinc-inducible E2A-HLF. Annexin II mRNA and protein expression persisted up to 32 hours after IL-3 starvation (Figure 4D), in contrast to the rapid decline in wild-type FL5.12 cells (Figure 3B), suggesting that E2A-HLF partially substitutes for the function of cytokine-initiated signaling pathways that induce annexin II expression.

Induction of cell-surface annexin II by E2A-HLF

To gain insight into the roles of annexin II overexpressed in leukemia cells with t(17;19), we quantified cell-surface annexin II by eluting it with calcium-free HBSS, which disrupts the calcium-dependent binding of annexin II to the cell surface (see “Materials and methods”). The eluate was then tested for annexin II by immunoblot analysis. As expected, no annexin II was detected when cells were treated with HBSS containing 4 mM CaCl₂ (Figure 5A, even-numbered lanes). Of 4 cell lines harboring the E2A-HLF chimeric protein, 3 showed high levels of surface annexin II, similar to that of NB-4 (lanes 1, 3, 7, and 19). In the third E2A-HLF–expressing cell line, HAL-O1, surface expression of annexin II was much less, though clearly, detectable (lane 5). Surface expression of annexin II was barely detectable from cells without E2A-HLF or PML-RARα (lanes 9, 11, 13, 15, and 17).

As a control, the eluates were also assayed for lactate dehydrogenase (LDH) activity. LDH activity was uniformly low in calcium-free and calcium-containing eluates (data not shown), suggesting that annexin II protein detected in this assay originated from the cell surface, not from the cytoplasm. Two cell lines with high levels of surface annexin II, YCUB-2 and Endo-kun, were derived from patients without hemorrhagic complications at onset, whereas HAL-O1, which expressed less surface annexin II, was established from a patient with coagulopathy (Table 1). These results thus suggested that levels of surface annexin II are not related to coagulopathy.

Annexin II mRNA and protein were quantified by densitometry; Figure 5B shows levels of mRNA and total and surface protein relative to those of NB-4 cells as positive control. The magnitude of
total annexin II protein induction was greater than that of mRNA, suggesting that E2A-HLF induces annexin II not only at the mRNA level but also through posttranscriptional mechanisms, in accordance with the results from enforced expression of E2A-HLF (Figure 2). In contrast, a comparison of the magnitude of induction of total annexin II protein with surface annexin II suggests that E2A-HLF does not selectively induce surface annexin II.

Next, we tested whether ectopic expression of E2A-HLF induces the surface expression of annexin II. Following zinc-induced overexpression of E2A-HLF in 697/E2A-HLF cells, levels of surface annexin II were increased (Figure 5C, lanes 1-4). Surface annexin II levels were unaffected by zinc in control 697/pMT cells, confirming that the observed changes in gene expression were induced by E2A-HLF and not by zinc (lanes 5-8).

**Lack of antiapoptotic activity of annexin II**

Because E2A-HLF protects IL-3-dependent lymphoid cells from apoptosis caused by IL-3 deprivation, we tested whether annexin II is involved in the regulation of cell survival (Figure 6A). FL5.12 cells expressing E2A-HLF survived more than 4 days in IL-3–free medium, as we previously reported (Figure 6B). In contrast, cells with zinc-induced overexpression of annexin II did not survive without the cytokine (Figure 6B), suggesting that annexin II does not contribute to the survival of hematopoietic progenitors.

**Discussion**

In this study, we demonstrated that annexin II expression is regulated by IL-3 in murine IL-3–dependent Baf-3 and FL5.12 cells (Figure 3). Using β544 cells and cells expressing constitutively active Ras mutants, Ras pathways, including Ras/PI3-K pathways, were shown to be major regulators of annexin II expression (Figure 4). On the other hand, enforced expression of E2A-HLF induced annexin II in these lymphoid cells in the absence of IL-3 (Figure 4D) and in human leukemia cell lines (Figure 2), indicating that annexin II is a downstream target of E2A-HLF. E2A-HLF induced annexin II not only by affecting mRNA levels but also through posttranscriptional mechanisms (Figures 2, 5B).

In earlier studies, we reported that inhibition of the DNA-binding ability of E2A-HLF by a dominant-negative form of this chimeric transcription factor induces apoptosis in UOC-B1 cells but does not affect the cell cycle. We also demonstrated that E2A-HLF protects Baf-3 and FL5.12 cells from apoptosis caused...
by IL-3 starvation, suggesting that E2A-HLF contributes to leukemogenesis through dysregulation of the cytokine-initiated cell survival system in hematopoietic progenitors. Consequently, we have postulated that a transcription factor acts as a physiologic counterpart of E2A-HLF in these IL-3-initiated cell survival systems. We identified a related bZIP factor, E4BP4/NFL3, as a candidate, because E4BP4 avidly binds to the consensus DNA-binding sequence of E2A-HLF and because E4BP4 is induced by IL-3 through signals mainly from the β chain distal portion, especially through Ras-Pi3-K and Ras-Raf-MAPK pathways. Moreover, the enforced expression of E4BP4 in IL-3-starved BaF-3 and FL5.12 cells delays apoptosis.

Annexin II expression is unlikely to be controlled by proapoptotic pathways regulated by E4BP4 because the overexpression of annexin II did not protect FL5.12 cells from apoptosis caused by IL-3 starvation (Figure 6B) and because we observed that the enforced expression of E4BP4 in IL-3-deprived FL5.12 cells did not induce annexin II (data not shown). Obviously, downstream targets of E4BP4 are not the only pathway that E2A-HLF aberrantly activates in B-precursor cells. E2A-HLF almost completely blocks apoptosis caused by cytokine deprivation of FL5.12 cells (Figure 6B), but E4BP4 has limited proapoptotic effects.

Therefore, annexin II appears to be regulated by another unidentified pathway under the control of IL-3 through Ras pathways in B-progenitor cells, and E2A-HLF constitutively activates this pathway to induce annexin II in t(17;19)–positive leukemia cells.

The proportion of annexin II on the cell surface compared with the total cellular expression levels varied among the cell lines. For instance, the total annexin II level in RS4;11 cells was nearly half that in NB-4 (Figure 1), but the cell surface annexin II of RS4;11 was barely detectable (Figure 5A), suggesting that translocation of this protein from the cytosol to the cell surface may be regulated in a manner dependent on cell lineage or maturation stage. The cell surface annexin II levels of 4 cell lines expressing E2A-HLF also diverged, in spite of the similar total annexin II levels of these 4 cell lines (Figures 1 and 5A-B). These results might be explained by the differences in E2A-HLF expressed in these 4 cell lines—that is, UOC-B1 expresses type 1, YCUB-2 and Endo-kun express type 2, and HAL-O1 expresses type 1 with a mutation in the leucine zipper region of HLF that alters the fusion protein’s DNA-binding properties.

The surface expression of annexin II is unlikely to be related to coagulopathy as an initial symptom, because HAL-O1 cells, which have low surface expression of annexin II, were derived from a patient with coagulopathy, whereas YCUB-2 and Endo-kun showed high expression of surface annexin II but derive from patients without coagulopathy (Table 1; Figure 5A). Surface annexin II could be correlated with hypercalcemia at onset, the other rare complication in pro-B ALL, because HAL-O1 is the only t(17;19)+ cell line that was derived from a patient without hypercalcemia. The biologic significance of cytokine–dependent annexin II expression in lymphoid cells is unclear, but it was recently reported that in rat adrenal pheochromocytoma (PC-12) cells, nerve growth factor (NGF) induces annexin II, which contributes to NGF-induced neuritogenesis in the differentiating PC-12 cells through the generation of plasmin.

On the other hand, annexin II has been implicated in the proliferation of hepatocytes and neurons and in the invasion and metastasis of various tumors, including glioblastoma multiforme, pancreatic cancer, lung cancer, and gastric cancer.

Annexin II interacts with procaspain B on the surfaces of tumor cells and is involved in extracellular proteolysis, facilitating tumor invasion and metastasis. It has also been suggested that annexin II may play a critical role in the tissue plasminogen activator–dependent, plasmin-mediated invasion of malignant glioma cells. Although overexpressed annexin II lacked antiapoptotic activity in IL-3–dependent cells (Figure 6B), E2A-HLF–positive leukemia is characterized by bone invasion and hypercalcemia, which are paraneoplastic syndromes that are rare complications in other types of childhood acute B-lineage leukemia. Based on the results of this study, we postulate that annexin II overexpression is a general feature of E2A-HLF–positive pro-B cell ALL and that it may have a causative role in one or more of the unique paraneoplastic syndromes associated with the expression of this oncogenic transcription factor.

Acknowledgments

We thank M. Eguchi for helpful discussions, support, and encouragement throughout this study. We thank F. J. Rauscher III for providing the pMT-CB6+ expression vector, A. Manabe for providing samples and information on patient 12, K. Harada and H. Aoyama for excellent technical assistance, and K. Ohyashiki and K. Toyama for the HAL-O1 cell lines.

References


Regulation of annexin II by cytokine-initiated signaling pathways and E2A-HLF oncoprotein

Takayuki Matsunaga, Toshiya Inaba, Hirotaka Matsui, Mayuko Okuya, Atsushi Miyajima, Takeshi Inukai, Tetsunori Funabiki, Mikiya Endo, A. Thomas Look and Hidemitsu Kurosawa

Updated information and services can be found at:
http://www.bloodjournal.org/content/103/8/3185.full.html

Articles on similar topics can be found in the following Blood collections
- Apoptosis (747 articles)
- Cell Adhesion and Motility (790 articles)
- Gene Expression (1086 articles)
- Hemostasis, Thrombosis, and Vascular Biology (2485 articles)
- Immunobiology (5496 articles)
- Neoplasia (4182 articles)
- Signal Transduction (1930 articles)

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml