ASB-2 Inhibits Growth and Promotes Commitment in Myeloid Leukemia Cells*

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In acute promyelocytic leukemia (APL) cells harboring the promyelocytic leukemia retinoic acid receptor α (PML-RARα) chimeric protein, retinoic acid (RA)-induced differentiation is triggered through a PML-RARα signaling resulting in activation of critical target genes. Induced differentiation of APL cells is always preceded by withdrawal from the cell cycle and commitment events leading to terminal differentiation. Here we have identified the human ankyrin repeat-containing protein with a suppressor of cytokine signaling box-2 (ASB-2) cDNA, as a novel RA-induced gene in APL cells. PML-RARα strongly enhanced RA-induced ASB-2 mRNA expression. In myeloid leukemia cells, ASB-2 expression induced growth inhibition and chromatin condensation recapitulating early events critical to RA-induced differentiation of APL cells.

Hematopoiesis is organized as a hierarchy of events articulated by both a genetic commitment and cytokines. When exposed to specific cytokines, hematopoietic progenitor cells divide to generate an amplified progeny and give rise to the various lineages (1). Several hematopoietic cytokines have been shown to be positive regulators of hematopoiesis. However, negative feedback is likely to be important in regulating signal transduction elicted by a large variety of extracellular signals. To this extent, suppressors of cytokine signaling (SOCS) proteins represent a family of feedback modulators of cytokine signal transduction that may play an important role in the regulation of normal hematopoiesis (2, 3). As in normal hematopoiesis, a hierarchy of events exists in leukemia hematopoiesis and it is assumed that leukemic cells descend from a very small pool of hematopoietic progenitors which are arrested at an immature stage of differentiation (4). It is expected that cytokine-responsive genes, which are critical to the maturation in normal hematopoietic progenitors, are repressed in those cells undergoing leukemic transformation, because of transcription factors and co-factors involved in chromosomal abnormalities (5, 6). Conversely, such genes should be induced in leukemia cells treated with inducers, such as retinoic acid (RA), which force de novo differentiation in leukemia cells. This should be the case in acute promyelocytic leukemia (APL) cells, which, as a result of a specific t(15;17) translocation, express the oncogenic PML-RARα chimeric protein and can undergo complete remission in patients treated with all-trans-retinoic acid (ATRA) which induces differentiation of the leukemic cells in vivo (7, 8). Retinoic acid receptors (RARs) are RA-dependent transcription factors (9) involved in the control of hematopoietic cells differentiation (10, 11). PML-RARα oncogenic effect is thought to involve transcriptional repression of RA target genes. In the presence of RA, PML-RARα activity is switched from repression to activation, acting as a RA-dependent enhancer factor (12). Several observations suggest that growth arrest and differentiation of APL cells by ATRA is triggered directly through a PML-RARα-dependent signaling pathway (12). In an early response to ATRA, PML-RARα-expressing cells undergo two rounds of cell division before entering postmitotic G1 phase of the cell cycle. Although post-mitotic G1 is a late event not crucially linked to the differentiation process, the G1 to S phase transition of the first cell cycle is required for the differentiation process to occur (13). Indeed, limitation in cell proliferative activity together with chromatin condensation represent a hallmark in commitment, an irreversible step committing hematopoietic cells to terminal differentiation (14). Furthermore, it is well established that this process is enacted in RA-induced differentiation of myeloid leukemia cells (15) and especially in APL, where ATRA induces growth arrest as well as chromatin condensation, which is characteristic of the emergence of polymorphonuclear leukocytes (16).

To identify RA target genes, which are critical to the process of growth arrest and differentiation, we have used differential screening with the NB4 acute promyelocytic leukemia cell line, which harbors the t(15;17) translocation encoding PML-RARα. These cells undergo granulocytic differentiation when treated with ATRA (17). We have identified and characterized the human ankyrin-repeat-containing protein with a SOCS box-2 (ASB-2) cDNA as induced by ATRA in NB4 cells. The ASB-2 mRNA was highly expressed in bone marrow cells. In ATRA-treated NB4 cells, increased ASB-2
mRNA expression correlated with the onset of myeloid differentiation. We demonstrate that, in ATRA-treated U937 myeloid cells, an increase in ASB-2 mRNA expression was mediated through an exogenously expressed PML-RARγ. Expression of an inducible exogenous ASB-2 in PLB-985 myeloid leukemia cells resulted in growth inhibition and chromatin condensation, which recapitulate early steps in induced differentiation of APL cells.

EXPERIMENTAL PROCEDURES

Leukemia Cells, Culture Conditions, Induction, and Assessment of Differentiation—Myeloblastic PLB-985 (18), promyelocytic NB4 (17), and RA-resistant subclones of NB4 cells (19) were kindly provided by W. Miller, M. Lanotte, and C. Nervi, respectively. U937-PR9 and U937-MT cells were generously provided by P. G. Pelicci (20). Cells were grown and used as described (21, 22). PLB-985/MT-ASB-2 and PLB-985/M5 MT cell lines were used for induction of ASB-2 expression or as control respectively. For this, cells were cultured either with or without ZnSO4. U937-MT and U937-PR9 cells were generated by stable transfection of the zinc-inducible sheep metallothionein promoter resulting in the pMT-ASB-2 vector. Exponentially growing PLB-985 cells (5 × 10⁶/ml of Opti-MEM (Invitrogen) were electroporated (Gene Pulser, Bio-Rad) at 300 V, 960 microfarads in the presence of 20 μg of the pMT-ASB-2 vector or the empty vector. Transfected PLB-985 cells were cultured for 48 h, selected with 0.4 g/ml G418 (Invitrogen), and cultured under limiting dilution conditions.

Northern Blot Analysis—Total RNA extraction and hybridization were as described (21). Human RNA master blot and human immune system multiple tissue Northern blot II were obtained from CLONTECH. The ASB-2 probe corresponded to the 3′ end of ASB-2 cDNA, respectively. Remaining single-stranded cDNAs were then separated by hydroxyapatite chromatography and used as specific probes. Two cDNA fragments (813 and 870) isolated from the RA-treated NB4 cDNA library contained 1007 bp (position 45–1051) and 1351 bp corresponding to the 3′ end of ASB-2 cDNA, respectively. A remaining 5′ cDNA sequence (44 bp) was amplified by rapid amplification of cDNA ends PCR from RA-treated NB4 RNA (with 5 × 10⁻⁷ M for 48 h) using the Marathon cDNA amplification kit (CLONTECH) with the primer: 5′-GCTCAGGCAGAAGCAGGAT-3′. Using the same kit, a contiguous cDNA was generated by reverse transcription-PCR amplification of ASB-2 from RA-treated NB4 cells.

In Vivo Expression—The ASB-2 coding sequence was subcloned into the pmTCBα-derived expression vector (25) under the control of the zinc-inducible sheep metallothionein promoter resulting in the pMT-ASB-2 vector. Exponentially growing PLB-985 cells (5 × 10⁶/ml of Opti-MEM (Invitrogen)) were electroporated (Gene Pulser, Bio-Rad) at 300 V, 960 microfarads in the presence of 20 μg of the pMT-ASB-2 vector or the empty vector. Transfected PLB-985 cells were cultured for 48 h, selected with 0.4 μg/ml G418 (Invitrogen), and cultured under limiting dilution conditions.

Southern Blot Analysis—Total RNA extraction and hybridization were as described (21). Human RNA master blot and human immune system multiple tissue Northern blot II were obtained from CLONTECH. The ASB-2 probe corresponded to the 3′ end of the 1351-bp ASB-2 cDNA. Radioactivity was quantified using a Storm 860 PhosphorImager (Amer sham Biosciences, Inc.).

Antibodies and Western Blot Analysis—An NH2-terminal peptide (SAPSRSSTAPPESSPA) was synthesized according to the ASB-2 deduced amino acid sequence and coupled to keyhole limpet hemocyanin through a cysteine residue added to the carboxyl-terminal amino acid of the peptide (Eurogentec). The rabbit serum (2PNA) was collected following the initial injection and three booster immunizations (Eurogentec). Immunoblot analyses were carried as described (22) except that the antisera was diluted 1:2000.

Staining of Cellular DNA, Cell Cycle Analysis—Cells were cytotype-unged onto microscope slides, fixed in 1% formaldehyde in ice-cold phosphate-buffered saline, and kept overnight at 4 °C in 70% ethanol. Cells were then stained with 5 μg/ml propidium iodide (PI; Molecular Probes) in phosphate-buffered saline containing 100 μg/ml DNase-free RNase A (Sigma) for 60 min and mounted under a coverslip in a solution of 9 parts of glycerol and 1 part of the PI staining solution.

Detection of Apoptotic Cells—Both in situ detection of DNA strand breaks by terminal dUTP nick-end labeling assay using the APO-BRDU kit (Phoenix Flow Systems) (26), and immunocytochemical detection of...
poly(ADP-ribose) polymerase cleavage product (27) were used for identification of apoptotic cells. Apoptotic cells were labeled with green fluorescing fluorescein isothiocyanate, and cellular DNA was counterstained with fluorescein red PI (28).

**Fluorescence Measurement**—Cellular red and green fluorescence intensities were measured by laser scanning cytometer (CompuCyte) as described (29, 30). DNA content frequency histograms were deconvoluted to obtain the percentage of cells in different phases of the cell cycle. The percentage of cells with condensed chromatin, i.e. with increased intensity of the DNA-associated maximal pixel PI fluorescence and decreased nuclear area, was obtained by gating analysis of the bivariate red integrated fluorescence versus maximal red fluorescence pixel distributions (29). The frequency of apoptotic cells was estimated from the bivariate distributions of PI versus fluorescein isothiocyanate integrated fluorescence intensities (28). At least 3000 cells were measured in each sample. The experiment was repeated three times.

**RESULTS**

**ASB-2 mRNA Is Up-regulated during RA-induced Granulocytic Differentiation of NB4 Cells**—Using subtractive hybridization, we have identified two novel cDNA clones (813 and 870) showing homology with the murine ASB-2 cDNA originally deduced from the analysis of contigs derived from expressed sequence tags (31). Increased ASB-2 mRNA expression occurred 4 h after exposure of NB4 cells to \(10^{-7}\)M ATRA with a maximum expression at 96 h (Fig. 1A). Sequential treatment of NB4 cells with cycloheximide and ATRA indicated that ATRA-induced ASB-2 mRNA up-regulation occurred even in the absence of de novo protein synthesis (Fig. 1A). Dose-response studies with ATRA-treated NB4 cells showed that 24 h of treatment with \(10^{-8}\) M ATRA induced peak levels of the...
ASB-2 mRNA (Fig. 1B), which correlated with differentiation as assessed by the NBT assay (data not shown). As expected, ATRA did not affect growth and differentiation (Fig. 1C, a and b, respectively) of NB4.306 cells that have lost the capacity to encode an intact ATRA-binding PML-RARα (19). In these cells, no induction of ASB-2 mRNA was observed (Fig. 1C, c). In contrast, ASB-2 mRNA was strongly up-regulated in NB4 cells induced to growth arrest and differentiation by ATRA (Fig. 1C). These data indicate that an increased ASB-2 mRNA expression correlated with the capacity of ATRA-treated leukemia cells to undergo growth arrest and granulocytic differentiation.

**ASB-2 Is a Member of the SOCS Family Highly Expressed in Human Bone Marrow**—We have cloned a 2396-bp cDNA (GenBank accession no. AY251238) (Fig. 2A) showing: (i) complete homology to a 1635-bp unpublished human sequence (GenBank accession no. AF159164) representing a partial human ASB-2 cDNA (positions 267–1907 in Fig. 2A) and (ii) 87% homology to a 1758-bp murine sequence (32) (GenBank accession no. AF155353) representing the complete coding sequence of the mouse ASB-2 cDNA (positions 144–1904 in Fig. 2A). Alignments of sequences from the 2396-bp ASB-2 cDNA, the partial human ASB-2, and the human chromosome 14 (GenBank accession no. AL079302) revealed some differences. Sequences of the 2396-bp ASB-2 cDNA and chromosome 14 were identical except an insertion of a G at position 1234, which was also found in partial ASB-2. This together with five 1-bp substitutions and two 3-bp insertions found in the 2396-bp ASB-2 cDNA when compared with the partial sequence indicates that the complete ASB-2 cDNA sequence reported here corresponds to the human ASB-2 mRNA. The complete open reading frame of ASB-2 consisted of 1764 bp translating into a 587-amino acid protein sharing 91% identity and 94% similarity with its mouse counterpart (data not shown). This protein harbors 11 ankyrin repeats and a SOCS box as defined by Michaely et al. (33) and Starr et al. (34), respectively. Ankyrin repeats contained 33 amino acids except repeats 2, 8, 9, and 10, which contained 34, 32, 42, and 30 amino acids, respectively (Fig. 2B). The ~50-amino acid SOCS box motif is composed of two well-conserved blocks of sequence, which are separated by 2–10 nonconserved residues (Fig. 2C). The carboxy-terminal conserved region is an leucine/proline-rich sequence of unknown function, and the amino-terminal conserved region is a 15-amino acid BC box known to interact with the elongin BC complex (35). In ATRA-treated NB4 cells, anti-ASB-2 polyclonal antibodies detected a specific 64-kDa protein after 4 h of treatment (Fig. 2D).

In the human immune system, ASB-2 mRNA was mainly expressed in bone marrow and to a lesser extent in spleen and lymph nodes (Fig. 2E). Using the Human RNA Master Blot, ASB-2 mRNA expression was also detected in skeletal muscle, heart, fetal heart, small intestine, appendix, bladder, aorta, stomach, uterus, prostate, and colon (data not shown). Our results indicated that ASB-2 is a member of the SOCS family relevant to normal hematopoiesis.

**Expression of an Exogenous ASB-2 Induces Growth Arrest and Chromatin Condensation in Myeloid Leukemia Cells**—To investigate whether induction of ASB-2 expression was involved in myeloid leukemia cell growth, an ASB-2 expression vector (MT-ASB-2) under the control of a zinc-inducible metallothionein promoter was stably transfected into PLB-885 cells. The MT vector was used as an empty control. When ZnSO₄ was added to the media, ASB-2 mRNA (Fig. 4A, a) and protein (Fig. 4A, b) expression increased dramatically in PLB-885/MT-ASB-2 cells. In the presence of ZnSO₄, a ~40% growth inhibition was observed in PLB-885/MT-ASB-2 cells whereas no significant change was detected in PLB-885/MT cells (Fig. 4B, a and b).

Laser scanning cytometer was used for measurements of cellular fluorescence intensity as assessment of DNA contents. Two representative samples of PLB-885/MT-ASB-2 cells, either untreated or treated with ZnSO₄ for 60 h, are shown in Fig. 4B (c and d, respectively). Based on the intensity of the DNA-associated red integrated fluorescence PI, we identified cells in G1 versus S and G2/M as shown on DNA content frequency histograms. Mitotic cells with condensed chromatids were distinguished from G2 cells by an increased intensity of maximal.
pixel of red fluorescence (Red Max Pixel) and by a decreased fluorescence area (29, 30). A large number of PLB-985/MT-ASB-2 cells treated with ZnSO₄ exhibited an increased intensity of maximal pixel of red fluorescence. After their relocation by laser scanning cytometer and examination by microscopy, they appeared to be interphase cells with smaller nuclei and condensed chromatin (Fig. 4B, d). The pattern of chromatin condensation in these cells did not resemble apoptosis-associated changes but rather was consistent with changes occurring in ATRA-induced HL-60 cells (29). We then analyzed the effect of ZnSO₄ on the DNA contents of PLB-985/MT-ASB-2 versus PLB-985/MT cells as well as the frequency of interphase cells with condensed chromatin. No change in proportions of untreated cells at different phases of the cell cycle or with condensed chromatin were detected (Fig. 4B, e and f). However, in the presence of ZnSO₄, few changes were observed with PLB-
985/MT cells (Fig. 4B, c), whereas the proportion of PLB-985/MT-ASB-2 cells with condensed chromatin was markedly increased (up to 35% at 60 h) (Fig. 4B, f). This was associated with an increased percentage of cells with a DNA content equivalent of that of S and G2/M cells (Fig. 4B, f). A large proportion of PLB-985/MT-ASB-2 cells with condensed chromatin had DNA content equivalent of that of S and G2/M cells (Fig. 4C, d). In separate measurements, we assessed the percentage of apoptotic cells with DNA strand breaks (terminal dUTP nick-end labeling-positive) (Fig. 4B, e and f) and cleaved poly-(ADP-ribose) polymerase (data not shown). The proportion of apoptotic cells remained below 5% whether PLB-985/MT-ASB-2 and PLB-985/MT were untreated or treated with ZnSO4. Thus, in the light of the evidence that proliferation of ASB-2 transfected cells was suppressed by ZnSO4 whereas apoptosis was minimal, the observed increase in proportion of S and G2/M cells including those cells with condensed chromatin indicates that, although commitment events were induced, cells were arrested in their progression through S and G2/M. However, expression of differentiation markers such as CD11b, ICAM-1, MCP-1, interleukin-8, and defensin 1 was not induced in H9251 models (43). Furthermore, expression of the PML-RAR of APL has been demonstrated in several transgenic mouse models (44). Whether ASB-2 expression is part of the mechanisms controlling growth arrest and chromatin condensation known to be associated with early phases of ATRA-induced differentiation of myeloid leukemia cells.

**DISCUSSION**

**ASB-2 Is a Member of the SOCS Protein Family**—ASB- proteins are evolutionarily conserved because translation of the M60–7 gene of the Caenorhabditis elegans cosmid M60 indicated that it encodes a full-length ASB (37) with bona fide ankyrin repeats and SOCS box organization. These proteins belong to the SOCS protein family identified as containing a SOCS box consensus (31). They differ in their NH2-terminal part, sharing sequences involved in protein-protein interactions such as a Src homology 2 domain (SOCS proteins), a SPRY domain (SSB proteins), WD-40 repeats (WSB proteins), or ankyrin repeats (ASB proteins). Several cytokines induce transcriptional activation of one or more SOCS genes, suggesting that SOCS proteins play central roles in determining the intensity and duration of cellular responses to many cytokines (3). Whether ASB-2 is involved in a specific cytokine signaling pathway remains to be determined.

**PML-RARα Enhances RA-induced Expression of ASB-2**—We found that the drastic increase in ASB-2 mRNA expression induced by RA in NB4 cells was mediated through a PML-RARα signaling and correlated with growth arrest and differentiation of these cells. Little is known about the signaling pathway through which pharmacological concentrations of RA can induce growth arrest and differentiation in APL cells. PML-RARα behaves as a constitutive transcriptional repressor of RA target genes in the presence of physiological concentrations of RA (58–41), and a central role of RARα pathway deregulation was found in the pathogenesis of APL (42). Therefore, PML-RARα may repress expression of ASB-2 and thus affect signaling at physiological concentrations of RA. The fact that RA concentrations can switch PML-RARα from repressor to activator (12) may account for a capacity of PML-RARα to transcriptionally activate the ASB-2 gene in the presence of RA. The causal role of PML-RARα in the pathogenesis of APL has been demonstrated in several transgenic mouse models (43). Furthermore, expression of the PML-RARα gene in murine bone marrow cells greatly blocked myeloid matura-

Because target cells for transformation in APL are likely to be myeloid progenitors (44), genes involved in myeloid differentiation might be repressed in APL cells and induced when these cells are exposed to RA. Our results suggest that ASB-2 may be one of these genes.

**ASB-2 Expression Recapitulates Early Differentiation Events in Myeloid Leukemia Cells**—In APL cells, RA induces growth arrest and terminal differentiation possibly via the induction of genes implicated in cell cycle arrest and the acquisition of a differentiated phenotype. Although, normal differentiation in most cell types is associated with an arrest in G1, a large proportion of the cells expressing an exogenous ASB-2 were actually arrested in the S and G2/M phases. This was quite evident from the presence of cells with condensed chromatin that had a S+G2/M DNA content and from the increase in overall proportion of S+G2/M cells after treatment with ZnSO4. During normal differentiation, the induction process appears to be sequential, first by arresting cells in G1 (by causing an accumulation of pRb in hypophosphorylated state) (45) and, subsequently, by inducing their differentiation phenotype. However, it should be noted that, in differentiating NB4 cells, hypophosphorylated pRb was found both in G1 and S+G2/M (46). In the case of ASB-2-expressing cells, the signaling appears to by-pass the cell cycle regulatory system and directly trigger commitment to differentiation. This observation is in agreement with the fact that, in PML-RARα-expressing cells exposed to the differentiative stimulus of RA, G1 arrest occurs as a late phase not causally linked to commitment and differentiation (13). It should also be noticed that, in uridine-induced HL-60 cells, an arrest in the G1 phase is not required for differentiation as this process is preceded by accumulation of cells in the G1/M phase of the cell cycle (47).

The fact that ASB-2 expression induced a limitation in cell proliferation together with chromatin condensation strongly suggests that it promotes commitment events found at early stages in RA-induced differentiation of leukemia cells. Further studies on the mechanisms of action of ASB-2 and the identification of its partners will contribute greatly to the understanding of the signaling pathway interrupted by PML-RARα at the early stages of leukemia transformation and reactivated during RA-induced commitment to differentiation in APL cells.

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**Addendum**—After this manuscript was submitted, Kohroki et al. reported the cloning of ASB-2 as induced by ATRA in HL-60 leukemia cells (Kohroki, J., Fujita, S., Itoh, N., Yamada, Y., Imai, H., Yumoto, N., Nakaniishi, T., and Tanaka, K. (2001) FEBS Lett. 505, 223–228)

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