Epigenetic Control of NF-κB-Dependent FAS Gene Transcription during Progression of Myelodysplastic Syndromes

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Abstract
The death domain containing TNF receptor 6 (CD95/Fas) is a direct target for the NF-κB transcription factor and is repressed in solid tumors such as colon carcinomas. Previously, we reported that the Fas death receptor, while overexpressed in low-risk myelodysplastic syndromes (MDS), becomes undetectable on CD34+ progenitors when the disease progresses to secondary acute myeloid leukemia (AML). This study determined the interplay between NF-κB and Fas during MDS progression. We first observed that Fas was induced by TNF-α in the HL60 cell line. In these cells, p65 (RELA) was associated with the FAS promoter, and inhibition of the NF-κB pathway by an IκBα inhibitor (BAY11-7082) or lentiviral expression of a nondegradable mutant of IκBα (IkSR) blocked Fas expression. In contrast, TNF-α failed to induce Fas expression in the colon carcinoma cell line SW480, due to hypermethylation of the FAS promoter. Azacitidine rescued p65 binding on FAS promoter in vitro, and subsequently Fas expression in SW480 cells. Furthermore, inhibition of the NF-κB pathway decreased the expression of Fas in MDS CD45+CD34+ bone marrow cells. However, despite the nuclear expression of p65, Fas was often low on CD45+CD34+ AML cells. TNF-α failed to stimulate its expression, while azacitidine efficiently rescued p65 binding and Fas reexpression. Overall, these data suggest that DNA methylation at NF-κB sites is responsible for FAS gene silencing. Mol Cancer Res; 11(7): 724–35. ©2013 AACR.

Introduction
CD95/Fas/APO-1/TNFRSF6 is a member of the death receptor family. Binding of its ligand FasL triggers the receptor trimerization and apoptosis signaling (1). This function is critical for the maintenance of hematopoietic cell homeostasis, in particular of the lymphocytic and erythroid lineages (2, 3). In pathologic conditions, suppression of Fas signaling leads to autoimmune diseases (4) and contributes to tumor-geneesis by inducing a resistance to FasL-dependent apoptosis (5). In established tumors with repressed Fas, restoration of Fas activity by transfection suppresses tumor growth and rescues apoptosis and chemosensitivity (5). Fas expression, which is regulated by TNF-α or IFN-γ in many cell systems including normal hematopoietic cells, is critically dependent on NF-κB activation (6, 7).

The transcription factor NF-κB, which functions as a homo or heterodimer composed of proteins p65 or p50, regulates a variety of cellular pathways that include inflammatory response, angiogenesis, proliferation, and survival (8). In resting cells, NF-κB is sequestered in the cytoplasm by an inhibitory IκB protein, predominantly IκBα (8). The canonical NF-κB pathway, which is typically activated upon TNF-α stimulation, involves the inhibitor of IκB kinase (IKK), leading to the proteosomal degradation of IκBα and the translocation of NF-κB to the nucleus (8–10). The constitutive activation of NF-κB pathway identified in solid tumors (11) and in diverse hematologic malignancies (12–16) has been suggested to promote cell survival through the transcription of apoptosis inhibitors such as BCL2L1 encoding BcXL or XIAP. However, NF-κB could also promote apoptosis in a cell type- and stimulus-dependent manner (17, 18), for example, through direct induction of FAS gene transcription in lymphocytes and liver cells (19, 20).

The transformation of myelodysplastic syndromes (MDS) to acute myelogenous leukemia (AML) is accompanied by the acquisition of mechanisms of resistance to apoptosis, either the upregulation of antiapoptotic proteins such as Bcl2 or the downregulation of proapoptotic effectors such as Bad.

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Although the forced expression of Bcl2 prevents MDS progenitor apoptosis in vitro (23), it has been recently shown using the MDS-like NUP98-HOXD13 transgenic mice model that Bcl2 does not stimulate the leukemic transformation (24). We have previously reported that Fas receptor was overexpressed at the cell surface in vitro of MDS CD34+ progenitors in early diseases, whereas it was downregulated at the time of progression (25). Furthermore, we also reported that FAS gene silencing in 60% of patients with secondary AML (sAML) was linked to the epigenetic repression of its transcription (26). The aim of the present work was to further investigate the interplay between FAS gene and NF-kB during the progression of MDS to AML.

Materials and Methods

Cell lines, reagents, and antibodies

HL60 and SW480 human cell lines were cultured in RPMI supplemented with glutamine, 10% fetal calf serum, penicillin–streptomycin (GibcoBRL Life Technologies). TNF-α was purchased from R&D Biosystem, BAY11-7082 and azacitidine were from Sigma-Aldrich. The antibodies used were as follows: CD45-PC5, CD34-PE, and CD95-FITC or isotypic IgG1-PC5, IgG1-PE, and IgG1-FITC (Beckman Coulter), p65/RelA rabbit polyclonal antibody (sc-372; Santa Cruz Biotechnology Inc.), and mouse monoclonal actin antibody (A5441; Sigma-Genosys), anti-rabbit and anti-mouse horseradish peroxidase–conjugated secondary antibodies (Cell Signaling Technology).

Bone marrow mononuclear or CD34+ cell isolation and culture

Forty-three patients with MDS, that is, 5q− syndrome (n = 1), refractory anemia (RA; n = 6), refractory cytopenia with multilineage dysplasia (RCMD; n = 4), RA or RCMD with ring sideroblasts (RARS or RCMD-RS; n = 9), RA with excess of blasts type I (RAEB1; n = 11), or type II (RAEB2; n = 12), or sAML (n = 10) and 15 age-matched controls with normal bone marrow were included between 2005 and 2011 after they gave their informed consent (Supplementary Table S1). This study was approved by the local ethics committee. CD34+ cells were sorted from mononuclear cell populations on magnetic beads (kit MACS; Miltenyi Biotech). Purity was controlled by flow cytometry and was always more than 85%. Mononuclear cells isolated on Ficoll gradient were maintained in Iscove’s modified Dulbecco’s medium (GibcoBRL Life Technologies) containing 10% fetal calf serum or 15% bovine serum albumin with insulin and transferrin, 50 ng/mL stem cell factor (SCF), 50 ng/mL thrombopoietin (TPO), 100 ng/mL Flt3-ligand (FLT3L) and 20 ng/mL interleukin-6 (IL-6). In some experiments, cells were incubated with 10 ng/mL of TNF-α.

Flow cytometry

Membrane Fas expression was quantified by flow cytometry on cell lines or bone marrow CD34+ cells population after red blood cell lysis and expressed as ratio of median fluorescence intensity (RFI) between specific to isotypic antibody. Analysis was conducted on FC500 flow cytometer (Beckman Coulter) using CXP Analysis software (Beckman Coulter).

Gene expression analysis

RNA from bone marrow mononuclear cell stored in Trizol (Invitrogen) or from cell lines was extracted on RNeasy Plus Mini kit (Qiagen). The quality was assessed by size fractionation by microfluidics instrument (Agilent Technologies). One microgram RNA was used per reverse transcription reaction using the Superscript2 (Invitrogen). cDNA strand was diluted and conserved at −80°C. Quantitative PCR (qPCR) was conducted with a LightCycler FastStart DNA Master PLUS SYBR Green I kit on Light Cycler (Roche Diagnostics). According to Minimum Information for Publication of Quantitative real-time PCR Experiments (MIQE) guidelines, a standard curve and an internal calibrator were prepared from U937 RNA. Each sample was tested twice for the expression of the transcripts and also for the expression of the reference genes GAPDH and B2M. The specific fluorescence threshold was the quantification cycle (Cq) value. Relative quantities (RQ) of FAS/TNFRSF6 or BCL2L1 were determined in patient or control samples compared with the calibrator (RQ = ΔCq,FAS/TNFRSF6 or BCL2L1/sample – calibrator) and normalized to reference genes as normalized relative quantity [NRQ = RQ,FAS/TNFRSF6 or BCL2L1/NF with NF = √(RQ,GAPDH × RQ,B2M)] according to the ΔCq method. Primer sequences and validation are summarized in Supplementary Table S2.

Immunofluorescence

Cells were washed in PBS 1× pH 7.4 and cytopsinized. After treatment with acetone, cell fixation was achieved with 2% PBS–formaldehyde for 20 minutes. Then, cells were permeabilized in methanol for 10 minutes at room temperature, followed by 15 minutes at 4°C with PBS-Trition 0.2%. After saturation in PBS containing 3% human serum albumin (HSA) for 1 hour, cells were incubated with p65/RelA rabbit polyclonal antibody at 1:200 in PBS–HSA 1%–TWEEN 0.1% overnight at 4°C. After washing, cells were incubated with goat anti-rabbit Alexa Fluor 568–conjugated antibody (Molecular Probes, Invitrogen) at 1:5,000 for 40 minutes at room temperature. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; Sigma) at 1:1,000 for 5 minutes at room temperature. Images were obtained on a Leica DMB microscope and analyzed using Metamorph software (Molecular Mevicer Corp.). Signals were quantified using ImageJ software (NIH, Bethesda, MD).

Nuclear extracts and p65/RelA oligoprecipitation

Cells were lysed for 10 minutes at 4°C in buffer A (10 mmol/L HEPES, pH 7.6, 3 mmol/L MgCl2, 10 mmol/L KCl, 5% glycerol, and 0.5% NP-40) containing 1 mmol/L Na2VO4, 20 mmol/L NaF, 1 mmol/L sodium pyrophosphate, 25 mmol/L β-glycerophosphate and proteinase inhibitors (Roche Diagnostics). After centrifugation, nuclear
pellets were extracted in buffer A containing 300 mmol/L KCl. For p65/RelA pulldown assays, nuclear extracts (10⁷ cells) were precipitated on 2 µg double-strand biotin-labeled oligonucleotide at 4°C for 1 hour. DNA–protein complexes were then pelleted using streptavidin–agarose beads (Amersham Biosciences). Beads were then washed 3 times with buffer A and suspended in 1× Laemmli buffer. Sequences of biotinylated oligonucleotides are in Supplementary Table S3.

**Western blot analysis**

Samples were subjected to 10% SDS–PAGE and transferred to nitrocellulose membrane. Filters were blocked overnight in 5% skimmed milk TBS pH 7.4 0.05% Tween 20 and incubated with the appropriate antibody. Membranes were washed 3 times in TBS-Tween 20 and incubated for 1 hour with the appropriate peroxidase-conjugated secondary antibody.

**Lentivirus construction and cell infection**

Plasmid encoding IκBα super-repressor (IkSR) provided by Dr. F. Porteu (Institut Cochin, Paris, France) was inserted in a TRIP ΔU3-EF1α defective lentivirus expressing the GFP under an IRES. CD34⁺ cells were infected twice at day 1, 5, and 1 after purification. Fas expression was quantified 3 days later on GFP-positive cells by flow cytometry.

**FAS promoter methylation analysis**

Sodium bisulfite treatment of genomic DNA was used to convert unmethylated cytosine to uracil by oxidative deamination. Briefly, after treatment with proteinase K, DNA was extracted from cell lines or bone marrow CD34⁷ progenitors using the DNA Extraction Kit BACC2 (GE Healthcare), treated in liquid phase by sodium bisulfite using Methyl Detector (Active Motif), dried, solubilized in RNase-free water, and stored at −80°C until use. CD34⁺ cells embedded in agarose beads were treated with 0.2 mg/mL proteinase K RNA grade (Invitrogen) overnight at 37°C in 10 mmol/L Tris–HCl pH 7.5, 10 mmol/L EDTA, 50 mmol/L NaCl, 0.2% SDS. Bead-coated DNA was denatured by heating and incubated for 4 hours at 37°C in a fresh solution of 2.8 mol/L sodium bisulfite, 0.5 mol/L hydroquinone, and 0.6 mol/L NaOH, pH 5.8. Beads transferred to a Multiscreen-HV (Millipore) filtration plate were washed 8 times in 10 mmol/L Tris–HCl pH 8.0, 10 mmol/L EDTA, and the reaction was stopped by 0.2 N NaOH. FAS promoter was amplified by PCR using the DNA Polymerase Taq Platinum Kit (Invitrogen). Primer pairs specific for bisulfite-treated genomic DNA are described in Supplementary Table S3. PCR products were purified using the QIAquick PCR Purification Kit. For bone marrow CD34⁷ samples, PCR products were cloned in bacteria using TOPO TA Cloning kit. Plasmid DNA was extracted and sequenced on ABI Prism 3100 apparatus (Applied Biosystems).

**Chromatin immunoprecipitation**

HL60, SW480 cells, or bone marrow mononuclear cells were cross-linked with 1% formaldehyde for 10 minutes at room temperature, lysed in 50 mmol/L Tris–HCl, pH 8.0 containing 1% SDS, 10 mmol/L EDTA, and sonicated with a Vibracell Sonifier (Bioblock Scientific). Chromatin immunoprecipitation (ChIP) experiments were carried out on solubilized chromatin extracted from 10 × 10⁶ cells and diluted 10-fold in ChIP dilution buffer (20%–50% Millipore). Chromatin was preselected for 1 hour and incubated overnight with 2 µg antibodies against acetyl histone H3 lysine 9/14 (H3K9/14ac), dimethyl histone H3 lysine 4 (H3K4me2), trimethyl histone H3 lysine 27 (H3K27me3) from Millipore, or dimethyl histone H3 lysine 9 (H3K9me2) from Abcam or 2 µg p65/RelA antibody or isotopic IgG1-mouse/rabbit (Santa Cruz Biotechnology). Immunocomplexes were collected on salmon sperm DNA/protein A agarose beads and washed. Modified histone/DNA complexes were eluted with 500 µL 1% SDS, 0.1 mol/L NaHCO₃. After cross-link reversal, DNA was purified by phenol extraction. An aliquot of the sonicated chromatin was treated identically for use as input. qPCR for FAS, NFκBIA, and B2M (Supplementary Table S3) was conducted with the following thermal cycling program: 5 minutes at 95°C, 40 cycles of 30 seconds at 95°C, 30 seconds at 60°C, and 45 seconds at 72°C, followed by a 5-minute extension time at 72°C. Relative enrichment of specific gene segment in ChIP was normalized to DNA input and control immunoglobulin G (IgG) and expressed as followed: 2^(ΔCq input–ΔCq gene)/2^(ΔCq input–ΔCq IgG).

**Luciferase assay for in vitro measurement of FAS promoter activity**

Reporter construction pGL2 FAS-Luc containing the −1,519 to +201 FAS promoter region spanning over the transcription start site (TSS; +1) was a gift from Dr. L.B. Owen-Schaub (MD Anderson Cancer Center, Houston, TX; ref. 19). Methylated plasmids were obtained by incubation of 5 µg of pGL2 FAS-Luc or empty pGL2 with CpG methyltransferase SssI (New England Biolabs) during 4 hours at 37°C. DNA methylation was checked by digestion with the methylation-sensitive restriction endonuclease HpaII. Methylated or unmethylated plasmids were cotransfected in 293T cells (5 × 10⁴) at 300 ng/well with pRL-tk Renilla vector at 10 ng/well to monitor transfection efficiency. At day 2 posttransfection, cells were lysed in Passive Lysis Buffer (Promega) for both firefly and Renilla luciferase activities measurement using the Dual Luciferase Reporter Assay (Promega). Normalized reporter activity is expressed as the firefly luciferase value to the Renilla luciferase value for each experimental condition.

**Results**

**The NF-κB inhibitor, BAY11-7082, inhibits Fas expression in HL60 cell line**

To explore the role of NF-κB in the regulation of FAS gene expression, we first compared the level of Fas receptor in various tumor cell lines. We chose the myelomonocytic cell line HL60 that expressed Fas, and the colon carcinoma cell line SW480 that did not express the receptor. TNF-α (10 ng/mL) increased the expression of Fas at the surface of HL60.
the HL60 cells after 6 hours, whereas it failed to induce Fas on SW480 cells. However, TNF-α (2 hours) stimulated the expression of another NF-κB target, BclxL, in these cells (Fig. 1A). Consistently, TNF-α upregulated the expression of FAS transcript in HL60, but not in SW480 cells, and BCL2L1 that encodes BclxL was upregulated after 2 hours of

**Figure 1.** p65/RelA inhibitor, BAY11-7082, inhibits Fas expression in HL60 cell line. A, kinetics of Fas expression upon TNF-α (10 ng/mL) stimulation in HL60 cell line (white bars) and kinetics of Fas (gray bars) and BclxL (hatched bars) in SW480 cell line by flow cytometry or by quantitative real-time PCR (qPCR). Results are expressed as a ratio of median fluorescence intensity (RFI) to isotypic control or NRQs of FAS or BCL2L1 to GAPDH, RNA18S and HPRT, respectively. B, immunolocalization of p65/RelA before and after 30 minutes of treatment with 10 ng/mL TNF-α in HL60 and SW480 cell lines. Nuclei were colored with DAPI. C, immunolocalization of p65/RelA in HL60 cells untreated (0) or treated with 5 μmol/L of BAY11-7082 for 2, 4, or 6 hours. Nuclei were colored with DAPI. D, one representative of 3 independent experiments. D, Western blot analysis for p65/RelA protein in nuclear and cytoplasmic extracts prepared from HL60 cell line, treated or not with 5 μmol/L of BAY11-7082 for 6 hours. Hsc70 is a loading control. E, kinetics of Fas expression upon treatment with 5 μmol/L of BAY11-7082 in HL60 cell line by qPCR or flow cytometry. Results are expressed as normalized relative quantities (NRQ) using the ΔCq method or as RFI, respectively. BCL2L1 gene was used as a positive control.
incubation with TNF-α in SW480 cells (Fig. 1A). p65/RelA subcellular localization was analyzed by immunofluorescence in both cell lines at baseline and after 30 minutes of incubation with 10 ng/mL of TNF-α. In HL60 cells, the p65/RelA protein was detectable in both the nucleus and the cytosol at baseline and after treatment with TNF-α (Fig. 1B). p65/RelA poorly present in the nucleus of SW480 cells, strongly relocalized to the nucleus after a 30-minute treatment with TNF-α and remained in the nucleus until 6 hours (data not shown). These data show that Fas remained unexpressed in SW480 cell line although the NF-κB pathway was activated as shown by p65/RelA nuclear localization and BCL2L1 transcription.

We then abrogated NF-κB canonical signaling pathway by treating the cells with a chemical inhibitor of IκBα phosphorylation and proteasomal degradation, BAY11-7082. p65/RelA disappeared from the nucleus upon exposure to 5 μmol/L BAY11-7082 (Fig. 1C). Immunoblot experiments confirmed the diminution of p65/RelA in the nucleus of BAY11-7082–treated cells (Fig. 1D). In the tested conditions, BAY11-7082–treated HL60 cells were devoid of any sign of apoptosis (not shown), and FAS transcript level decreased with time (Fig. 1E, left). BAY11-7082 also reduced, although less efficiently, the expression of BCL2L1 transcript (Fig. 1E, left) and decreased the expression of Fas at the cell surface (Fig. 1E, right).

p65/RelA binds to FAS promoter

The FAS gene promoter contains 3 GGGRNYYCC putative NF-κB sites with R = A/G and Y = T/C in the 5′-untranslated region (5′-UTR) region spreading between −899 and the TSS (+1; Fig. 2A; ref. 19). To determine whether p65/RelA could be recruited at these sites, we carried out in vitro DNA affinity precipitation experiments with nuclear extracts prepared from HL60 cells treated with 5 μmol/L BAY 11-7082 for 6 hours. p65/RelA was precipitated by using 30-bp biotinylated oligonucleotides whose sequence contained either one of the 3 putative NF-κB–binding sites of the FAS promoter, or a consensus tandem NF-κB–binding site as a positive control, or a...
consensus GFI-1B–binding site as a negative control. As shown in Fig. 2B, p65/RelA bound to each of the 3 putative NF-kB–binding sites on FAS promoter, which was prevented by BAY11-7082. Using ChIP assays (Fig. 2C), we found that p65/RelA spontaneously bound to the FAS promoter in HL60 cells. p65/RelA was not recruited at the FAS promoter in Fas-negative SW480 cells. As controls, p65/RelA was recruited to NFKBIA gene promoter that contains a NF-kB site, but not to B2M promoter that does not have any NF-kB site, both in HL60 or SW480 cells (Fig. 2C). Thus, p65/RelA binds to the chromatin at FAS promoter in a cell type–dependent manner.

FAS gene expression is epigenetically regulated

To explain why the recruitment of p65/RelA to the chromatin varied depending on the cell type, we hypothesized that a closed conformation of the chromatin in the FAS gene 5’–UTR regulatory regions may prevent the docking of NF-kB. To address this question, we compared the DNA methylation level at FAS promoter using bisulfite-treated genomic DNA–specific PCR. The promoter region of human FAS gene spanning in the 5’–UTR region comprised between −899 and +231 contains 35 CpG dinucleotides, of which 26 belong to a CpG island (Methyl Primer Express software v1.0, Applied Biosystems; Fig. 3A). Although only
A

At diagnosis
After progression

RFI Fas

% BM blasts

50
40
30
20
10
0

51 11 14 9 24 22 28 4 12 8 32 16 40 41 3 21 49 17 34 35 36 10

B

sAML# 3 17 35 49

p65/RelA

p65/RelA

DAPI

TNF-α (6 h) – + – +

NRQ

0 2 4 6 8 10

FAS BCL2L1 FAS BCL2L1 FAS BCL2L1 FAS BCL2L1

RFI Fas

RFI BclxL

TNF-α (6 h) – + – + – +

R² = 0.2158

% BM blasts

0 10 20 30 40 50

0 10 20 30 40 50
2 of 35 CpG (6%) were methylated in the Fas-positive HL60 cell line, 24 of 35 CpG (68%) located both 5′ upstream of and within the CpG island were methylated in the Fas-negative SW480 cells. In addition, ChIP experiments identified one active mark H3K9/14Ac and none of the 2 repressive marks H3K27me3 and H3K9me2 in HL60 cell line, whereas the repressive mark H3K9me2 was expressed in SW480 cell line (Fig. 3B). These results suggested that the chromatin conformation at FAS promoter was open in the Fas-positive HL60 cell line and repressed in the Fas-negative SW480 cell line. Treatment for 3 days with 1 µmol/L azacitidine induced the demethylation of 20 of 24 CpGs in the SW480 cells, whereas it did not influence the CpG methylation in HL60 cells (Fig. 3A). In these conditions, azacitidine did not induce cell apoptosis or necrosis (not shown) but clearly stimulated the expression of Fas in SW480 but not in HL60 cells (Fig. 3C, left and middle). Azacitidine also increased the recruitment of p65/RelA at FAS promoter in SW480 cell line, whereas it did not modify p65/RelA binding in the HL60 cell line (Fig. 3C, right). We also transfected 293T cells with a pGL2 construct containing the FAS promoter region (−1,519 to +201 over the TSS) in either a methylated or an unmethylated conformation, inserted upstream of the luciferase gene reporter (19). As shown in Fig. 3D, the luciferase activity was increased in cells transfected with the unmethylated FAS promoter construct, thus confirming the regulatory role of DNA methylation on FAS promoter activity. TNF-α (10 ng/mL for 6 hours) further increased the promoter activity that was abrogated by the NF-κB inhibitor, BAY11-7082. Altogether, NF-κB−dependent FAS gene expression is epigenetically regulated.

**NF-κB controls Fas expression in MDS/sAML bone marrow mononuclear cells**

The clinical relevance of these findings was investigated in primary bone marrow myeloid cells collected from 53 patients with MDS (n = 43) or sAML (n = 10) and 15 healthy controls. Karyotype was unavailable in 7 patients. According to the International Prognosis Scoring System (IPSS), 27 patients with MDS had a low-risk (LR)-MDS and 11 had a high-risk (HR)-MDS (Supplementary Table S1). In agreement with our previous data (26), the median Fas protein expression on primary bone marrow samples from MDS or AML was elevated mostly in patients with percentages of bone marrow blasts lower than 10%. Among those cases, 14 had a significant reduction of Fas level at the time of disease progression to HR-MDS or sAML, except for cases 14 and 28. The Fas RFI was inversely correlated with the percentage of bone marrow blasts.

We then investigated the effect of TNF-α on Fas expression. Primary bone marrow mononuclear cells isolated from 4 Fas-negative samples of AML were incubated with 10 ng/mL TNF-α for 6 hours. Immunolocalization of p65/RelA was conducted and showed the presence of p65/RelA in the nucleus of blast cells at baseline. After treatment with TNF-α, we observed an increment of the nuclear expression of p65/RelA in 3 cases (#3, 17, and 49), suggesting that NF-κB pathway could be activated in these cells (Fig. 4B). However, Fas expression remained low after treatment with TNF-α in the 4 cases both at transcript and protein levels, whereas BclxL expression increased in the same 3 cases (Fig. 4B). This suggests that TNF-α–stimulated transcription of FAS gene could be specifically abrogated in primary AML cells.

**BAY11-7082 decreases Fas expression on MDS/AML bone marrow mononuclear cells**

To investigate the role of NF-κB in the regulation of FAS gene transcription, we treated bone marrow mononuclear cells from patients with low/int-1 MDS (n = 11) or HR-MDS/AML (n = 10) with BAY11-7082 for 6 hours. Sixteen samples were positive for Fas before treatment and BAY11-7082 decreased the expression of Fas in 12 of them (Fig. 5A). We then investigated the subcellular localization of p65/RelA before and after BAY11-7082 treatment in 4 LR-MDS (2 RA, 1 RCM-D-RS, and 1, RAEB1) and 2 HR-MDS (1 sAML and 1 RAEB1 with a complex karyotype). Figure 5B shows 2 representative samples. p65/RelA was detected in the nucleus and in the cytoplasm and disappeared from the nucleus upon treatment with BAY11-7082 that also induced a decrease of FAS transcript expression in bone marrow cells (see 3 of the tested cases in Fig. 5C, top). In one case (#11), the sample size allowed following the kinetic of FAS and BCL2L1 gene repression (Fig. 5C, bottom). In addition, lentivirus-mediated expression of an iKsr in MDS CD34+ progenitors (n = 3) retained p65/RelA in the cytoplasm, which was associated with a decreased expression of Fas at the cell surface (Fig. 5D). This suggests that NF-κB may participate to the regulation of the transcription of FAS gene in primary bone marrow samples from MDS or AML.

**Azacitidine rescues p65/RelA–induced Fas expression in primary MDS/sAML cells**

In primary bone marrow mononuclear cells, we used ChIP to explore the binding of p65/RelA to the chromatin at FAS

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**Figure 4.** Fas expression on CD45+CD34+ MDS/AML cells is inversely correlated to the bone marrow (BM) blast count and remains low in AML cells after treatment with TNF-α. A, top, flow cytometry analysis of membrane Fas expression on CD45+CD34+ bone marrow cells in 22 patients with MDS (19 LR-MDS and 3 HR-MDS) at diagnosis (white bars) and at the time of progression (black bars). Results are expressed as RFI to isotypic control. Middle, bone marrow blast percentages. Right, inverse correlation between Fas expression and percentage of bone marrow blasts. B, AML blasts (n = 4) were treated for 6 hours with TNF-α (10 ng/mL) before study of p65/RelA subcellular localization by immunofluorescence, quantification of FAS or BCL2L1 transcripts by quantitative real-time PCR (qPCR), without TNF-α (white bars), with TNF-α (hatched bars), and Fas or BclxL proteins measurement by flow cytometry. Results are expressed as NRQs or as RFI, respectively.
promoter in 4 Fas-expressing (3 RAEB1 and 1 RAEB2) and 6 Fas-negative (1 RA, 1 RCMD-RS, and 4 sAML) MDS samples (threshold RFI = 1.7). We observed that the enrichment of p65/RelA at FAS promoter was null or very low in the 4 sAML and in 1 RCMD-RS, which did not express Fas, and was positive in all 4 Fas-positive LR-MDS. In one case, (RA#2), the expression of Fas was at the threshold of positivity in accordance with a poor binding of p65/RelA. Our data suggest that p65/RelA binding at FAS promoter is low in HR-MDS/AML. Although the number of samples available for ChIP experiment was limited, we found that the expression of Fas was proportional to the recruitment of p65/RelA at the gene promoter (Fig. 6A; R² = 0.765).

We have previously shown that DNA methylation of the FAS gene promoter correlated with low Fas expression in sAML, and that this promoter can be demethylated in patients with MDS/AML treated with azacitidine (26). Here, bone marrow mononuclear or CD34⁺ cells from patients with sAML (n = 11) were cultured in vitro in the presence of TPO, IL-6, SCF, and FLT3L for 72 hours with or without 1 μmol/L azacitidine. In these conditions, cells did not differentiate nor they died (not shown). Azacitidine efficiently rescued Fas receptor expression on bone marrow CD45lo/CD34⁺ blast cells in the 7 Fas-negative cases, whereas the effect was weak in 2 Fas-positive cases (#14 and #47) and null in another Fas-positive case (#13; Fig. 6B). We then measured the methylation level of the CpGs in the −899 and +231 FAS promoter region in the purified CD34⁺ progenitors before and after azacitidine treatment for 2 patients. As shown in a representative experiment, azacitidine induced the demethylation of 14 of 16 CpG, including those located within NF-kB–binding sites 1 and 3 (Fig. 6C). Second, we tested the effect of azacitidine on the binding of p65/RelA to

Figure 5. BAY11-7082 decreases Fas expression in MDS/AML CD34⁺ or mononuclear cells. A, flow cytometry analysis of membrane Fas expression in MDS/sAML CD34⁺ or mononuclear cells before and after treatment with 5 μmol/L BAY11-7082 for 6 hours. Results are expressed as a RFI to isotypic control. B, inhibition of p65/RelA nuclear localization by BAY11-7082 (5 μmol/L for 6 hours) in MDS/AML (n = 6) mononuclear cells. Representative fluorescence microphotographs of p65/RelA labeling in 1 LR-MDS and 1 sAML. Nuclei are colored with DAPI. C, quantitative real-time PCR (qRT-PCR) for FAS and BCL2L1 gene expression in MDS mononuclear cells treated or not with 5 μmol/L of BAY11-7082 expressed as NRQ to GAPDH and B2M according to the ΔCq method. D, NF-κB pathway inhibition by lentiviral expression of IκS in MDS CD34⁺ cells. Infected cells were maintained for 3 days in cultures and then GFP⁺ cells were sorted and analyzed for p65/RelA subcellular localization by immunofluorescence (left) and Fas expression by flow cytometry (right). Results representative of 3 independent experiments.

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RelA binding and Discussion

sAML, CpG methylation of NF-FAS gene expression in hematopoietic progenitor cells. In therapy, leading toingly, this downregulation is reversible upon azacitidine account for the gene and protein downregulation. Interest-

promoter prevents p65/RelA binding to chromatin, which

marrow CD45loCD34 samples. Results are expressed as a RFI to isotypic control. C, CpG methylation analysis. MDS/sAML CD34 (10 clones/sample). Black circles, fully methylated CpGs; open circles, unmethylated CpGs. D, ChIP of p65/RelA at

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induce mononuclear cells (Fig. 6D). Altogether, azacitidine can

scription factor at demethylation may facilitate the docking of NF-
p65/RelA recruitment after treatment, suggesting that DNA

promoter in one case of sAML (#23) the before and after

azacitidine treatment. We observed a dramatic increase of

p65/RelA compared with DNA input and control isotypic IgG. Middle, ChIP of p65/RelA at the

FAS promoter in primary bone marrow samples. Enrichment of p65/RelA compared with DNA input and control isotypic IgG. Top, correlation with Fas expression by

ow cytometry. B, ow cytometry analysis of Fas receptor on bone

marrow CD45loCD34 progenitors before (white bars) and after (black bars) in vitro treatment with 1 μmol/L azacitidine for 3 days in 11 MDS/sAML samples. Results are expressed as a RFI to isotypic control. C, CpG methylation analysis. MDS/sAML CD34− cells (n = 2) were treated for 3 days with 1 μmol/L azacitidine. Fas promoter was amplified on bisulfi
treated DNA from CD34− cells. Amplicons were cloned in bacteria and sequenced (10 clones/sample). Black circles, fully methylated CpGs; open circles, unmethylated CpGs. D, ChIP of p65/RelA at FAS promoter (n = 1). Enrichment of p65/RelA compared with DNA input and control isotypic IgG.

FAS promoter in one case of sAML (#23) the before and after

azacitidine treatment. We observed a dramatic increase of

p65/RelA recruitment after treatment, suggesting that DNA demethylation may facilitate the docking of NF-κB transcrip-
tion factor at FAS promoter in primary bone marrow mononuclear cells (Fig. 6D). Altogether, azacitidine can induce FAS promoter demethylation, thus facilitates p65/RelA binding and FAS gene transcription in myeloid cells.

Discussion

The present study shows that NF-κB directly promotes FAS gene expression in hematopoietic progenitor cells. In sAML, CpG methylation of NF-κB–binding sites at FAS promoter prevents p65/RelA binding to chromatin, which account for the gene and protein downregulation. Interestingly, this downregulation is reversible upon azacitidine therapy, leading to FAS gene reexpression.

NF-κB can promote cell survival through the transcriptional regulation of IAP (XIAP, cIAP1, and cIAP2) or BclXL (BCL2L1) family members or cell proliferation through the upregulation of CYCD1 and c-MYC (16, 27). NF-κB can also facilitate cell death through the death receptor–mediated extrinsic pathway (19, 20, 27). These dual pro- and antiapoptotic functions of NF-κB could apply to MDS. In LR-MDS, the NF-κB activity, which could be due to TNF-α secretion (28–31), may account for FAS gene expression. NF-κB pathway is constitutively activated when the disease progresses (12–16) and could suppress cell death through the transcriptional regulation of antiapoptotic genes. In addition, the methylation of promoters could turn off the NF-κB—dependent transcription of proapoptotic genes such as FAS (32).

The present study shows the direct upregulation of FAS gene expression by NF-κB in primary bone marrow cells. p65/RelA binds to the FAS gene promoter, whereas NF-κB inhibition with either BAY11-7082 (33) or the IkSR decreases Fas expression. The FAS gene has been shown previously to be a direct target for NF-κB in lymphocytes,
hepatocytes (6, 20), and various solid tumor cell types (11, 32). The cell context may be essential as NF-κB activation through the alternate pathway could be a FAS transcription repressor in murine erythroleukemia (MEL) cells (34). Alternatively, the threshold of NF-κB activity, which is under control of either the quantity of dimers p65/p50, p50/p50, and p65/p50 recruited to the chromatin or posttranslational modifications, or interactions with other transcription factors such as Sp1 could determine its effects (35, 36). For instance, a high rate of activation could permit the transcription of a large spectrum of pro and antiapoptotic genes, low levels of activation could restrict the transcription to few proapoptotic genes. In our model of myeloid leukemogenesis, MDS at the beginning are characterized by inappropriate and excessive apoptosis linked to the upregulation of FAS gene expression. At this stage, NF-κB activation is weak while it increases as the disease progresses to AML together with the upregulation of anti-apoptotic genes such as cIAPs, Bcl2 or downregulation of proapoptotic gene such as FADD (16, 21, 37). In addition, the NF-κB consensus binding sequences play an important role in determining the specificity of the different active dimers and affect their interaction with transcription coactivators (38). This may suggest that either the threshold or the specificity of NF-κB pathway could be crucial for cell fate decision between survival and death. Finally, the cell fate could depend on the epigenetic state of the chromatin at NF-κB target gene promoters. Our previous and current data support this later hypothesis as FAS gene expression decreases along the progression of MDS to AML according to the increase of DNA methylation at FAS gene promoter (26). The FAS gene expression is also epigenetically regulated in neoplastic mast cells in which azacitidine or decitabine treatment induced caspase-dependent apoptosis through the reactivation of this gene (39).

In the FAS promoter of the colon carcinoma cell line SW480 or AML primary samples (22) methylated CpG are located in the CpG island around the TSS (−285 to +221) and in a 5′ region upstream of the CpG island (−899 to −286), both regions containing NF-κB putative binding sites for transcription factors. Consequently, the binding of p65/RelA to chromatin is very low. Treatment with azacitidine rescues Fas expression, both at the transcript and protein levels by facilitating the binding of p65/RelA to the chromatin. p65/RelA binding to the chromatin is more important in low-grade MDS where the CpG methylation is low compared with high-grade MDS in which the level of DNA methylation is high. Such an epigenetic regulation of NF-κB—dependent transcription has been reported for AIOLOS gene in chronic lymphocytic leukemia (40).

Altogether, CpG methylation inhibits the binding of p65/RelA to FAS promoter and decreases FAS gene transcription in high-grade MDS and sAML, which may contribute to cell resistance to apoptosis and disease progression. The increase in the expression of FAS gene that we observed during the clinical use of azacitidine in 63 patients with MDS/AML could be used as a biomarker of the drug activity (26). It will be useful to determine whether this reexpression of Fas contributes to slow down the progression of the disease or constitutes a potential risk of treatment failure in a prospective clinical trial.

Disclosure of Potential Conflicts of Interest
M. Fontenay is a consultant/advisory board member of Celgene. No potential conflicts of interest were disclosed by the other authors.

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References
1. Lavrik IN, Krammer PH. Regulation of CD95/Fas at the DISC. Cell Death Differ 2012;19:36–41.

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Epigenetic Control of NF-κB-Dependent FAS Gene Transcription during Progression of Myelodysplastic Syndromes

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