Epigenetic control of NFκB dependent FAS gene transcription during progression of myelodysplastic syndromes

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Abstract

*FAS* gene, a direct target for NFκB transcription factor, is repressed in solid tumors including colon carcinomas. We have previously reported that, while overexpressed in low risk myelodysplastic syndromes (MDS), the Fas death receptor becomes undetectable on CD34⁺ progenitors when the disease progresses to secondary acute myeloid leukaemia (sAML). This study aimed at investigating the interplay between NFκB and Fas during MDS progression.

We first observed that Fas was inducible by TNFα in the HL60 cell line. In these cells, p65/RelA bound to the chromatin at *FAS* promoter, while inhibition of NFκB pathway by IKKα inhibitor BAY11-7082 or lentiviral expression of a non-degradable mutant of IκBα, IκSR blocked the expression of Fas. By contrast, TNFα failed to induce Fas expression in the colon carcinoma cell line SW480, due to hypermethylation of the *FAS* promoter. *In vitro* use of azacitidine rescued p65/RelA binding on *FAS* promoter and, subsequently Fas expression in SW480 cells. We also show that, inhibition of NFκB pathway decreased the expression of Fas in MDS CD45loCD34⁺ bone marrow cells. However, despite of the nuclear expression of p65/RelA, Fas was often low on CD45loCD34⁺ AML cells. TNFα failed to stimulate its expression, while azacitidine efficiently rescued p65/RelA binding and Fas re-expression. Our data suggest that DNA methylation at NFκB sites is responsible for *FAS* gene silencing. Rescuing *FAS* gene expression by azacitidine could contribute to slacken the progression to leukaemia.
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 pathological conditions, suppression of Fas signaling leads to autoimmune diseases (4), and contributes to tumorigenesis 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 IFNγ in many cell systems including normal hematopoietic cells, is critically dependent on nuclear factor (NF)κB activation (6, 7).

The transcription factor NFκB, 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, NFκB is sequestered in the cytoplasm by an inhibitory IκB protein, predominantly IκBα (8). The canonical NFκB pathway, which is typically activated upon TNFα stimulation, involves the IκB kinase complex (IKK), leading to the proteasomal degradation of IκBα and the translocation of NFκB to the nucleus (8, 9, 10). The constitutive activation of NFκB pathway identified in solid tumors (11) and in diverse hematological malignancies (12-16), has been suggested to promote cell survival through the transcription of apoptosis inhibitors like BCL2L1 encoding BclXL or XIAP. However, NF-κB could also promote apoptosis in a cell type- and stimulus-dependent manner (17, 18), e.g. through direct induction of FAS gene transcription in lymphocytes and liver cells (19, 20).
The transformation of MDS to AML is accompanied by the acquisition of mechanisms of resistance to apoptosis, either the upregulation of anti-apoptotic proteins like Bcl2 or the downregulation of pro-apoptotic effectors like Bad (21,22). While 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 of MDS CD34+ progenitors in early diseases while it was downregulated at the time of progression (25). Furthermore, we also reported that FAS gene silencing in 60% of patients with secondary AML 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 NFκB during the progression of MDS to AML.

**Material and methods**

**Cell lines, reagents and antibodies**

HL-60 and SW-480 human cell lines were cultured in RPMI supplemented with glutamine, 10% fetal calf serum, penicillin-streptomycin (GibcoBRL Life Technologies, Paisley, UK). Tumor necrosis factor-α (TNF-α) was purchased from R&D Biosystem (Minneapolis, MN), BAY11-7082 and azacitidine were from Sigma Aldrich (Saint Louis, MO). The antibodies used were as follows: CD45-PC5, CD34-PE and CD95-FITC or isotypic IgG1-PC5, IgG1-PE and IgG1-FITC (Beckman Coulter, Miami, FL), p65/RelA rabbit polyclonal antibody (sc-372, Santa Cruz Biotechnology Inc., Santa Cruz, CA), and mouse monoclonal actin antibody (A5441, Sigma-Genosys, Cambridge, U.K.), anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Beverly, MA).

**Bone marrow mononuclear or CD34+ cell isolation and culture**

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Forty-three patients with a MDS *ie* 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 1 (RAEB1, n=11), or type 2 (RAEB2, n=12), or secondary AML (sAML, n=10) and 15 age-matched controls with normal bone marrow (BM) were included between 2005 and 2011, after they gave their informed consent (*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, Bergisch Badgach, Germany). Purity was controlled by flow cytometry and was always > 85%. Mononuclear cells isolated on Ficoll gradient were maintained in Iscove’s modification of Dulbecco medium (GibcoBRL Life Technologies) containing 10% fetal calf serum or 15% bovine serum albumin with insulin and transferrin, 50ng/mL stem cell factor (SCF), 50ng/mL thrombopoietin (TPO), 100ng/mL Flt3-ligand (FLT3L) and 20ng/mL interleukin-6 (IL-6). In some experiments, cells were incubated with 10 ng/mL of TNFα.

**Flow cytometry**

Membrane Fas expression was quantified by flow cytometry on cell lines or bone marrow CD45lo/CD34+ cell population after red blood cell lysis and expressed as ratio of median fluorescence intensity (RFI) between specific to isotypic antibody. Analysis was performed on FC500™ flow cytometer (Beckman Coulter, Miami, FL) using CXP Analysis software (Beckman Coulter).

**Gene expression analysis**

RNA from BM mononuclear cell stored in Trizol® (Invitrogen, Carlsbad, CA) or from cell lines was extracted on RNeasy Plus Mini kit (Qiagen, Valencia, CA). The quality was assessed by size fractionation by microfluidics instrument (Agilent Technologies, Palo Alto, CA). One μg RNA was used per reverse transcription reaction using the Superscript2 (Invitrogen). cDNA strand was diluted and conserved at -80°C. Quantitative PCR (qPCR) was performed with a LightCycler® FastStart DNA Master PLUS SYBR® Green I kit on Light
Cycler® (Roche Diagnostics, Mannheim, Germany). According to 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 to the calibrator $U_{937}$ [RQ = $\Delta$Cq$_{FAS/TNFRSF6}$ or $BCL2L1$ (sample-calibrator)] and normalized to reference genes as normalized relative quantity [NRQ = $RQ_{FAS/TNFRSF6}$ or $BCL2L1$/$\sqrt{RQ_{GAPDH} \times RQ_{B2M}}$] according to the $\Delta$Cq method. Primer sequences and validation are summarized in Table S2.

**Immunofluorescence.**

Cells were washed in phosphate-buffered saline (PBS) 1X pH 7.4 and cytopspined. After treatment with acetone, cell fixation was achieved with 2% PBS-formaldehyde for 20 min. Then, cells were permeabilized in methanol for 10 min at room temperature, followed by 15 min at 4°C with PBS-Triton 0.2%. After saturation in PBS containing 3% human serum albumin (HSA) for 1 h, cells were incubated with p65/RelA rabbit polyclonal antibody at 1:200 in PBS-HSA 1%-Tween 0.1% over night at 4°C. After washing, cells were incubated with goat anti-rabbit Alexa Fluor 568-conjugated antibody (Molecular Probes, Invitrogen, Carlsbad, CA) at 1:5000 for 40 min at room temperature. Nuclei were stained with diaminido phenylindol (DAPI, Sigma) at 1:1000 for 5 min at room temperature. Images were obtained on a Leica DMB microscope and analyzed using Metamorph software (Molecular Devices Corp, Dowington, PA). Signals were quantified using ImageJ software (NIH, Bethesda, MY).

**Nuclear Extracts and p65/RelA oligoprecipitation**

Cells were lysed for 10 min at 4°C in buffer A (10 mM HEPES, pH 7.6, 3 mM MgCl$_2$, 10 mM KCl, 5% glycerol, 0.5% NP-40) containing 1 mM Na$_2$VO$_4$, 20 mM NaF, 1 mM sodium pyrophosphate, 25 mM β-glycerophosphate and proteinase inhibitors (Roche Diagnostics, Mannheim, Germany). After centrifugation, nuclear pellets were extracted in buffer A
containing 300 mM KCl. For p65/RelA pulldown assays, nuclear extracts (10^7 cells) were precipitated on 2μg double-strand biotin-labeled oligonucleotide at 4°C for 1 hour. DNA-protein complexes were then pelleted using streptavidin-agarose beads (Amersham Biosciences, Piscataway, NJ). Beads were then washed three times with buffer A and suspended in 1× Laemmli buffer. Sequences of biotinylated oligonucleotides are in Table S3.

**Western Blot Analysis**

Samples were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Filters were blocked overnight in 5% skimmed milk Tris-buffered saline (TBS) pH7.4 0.05% Tween 20 and incubated with the appropriate antibody. Membranes were washed three times in TBS-Tween 20 and incubated for 1 hour with the appropriate peroxidase-conjugated secondary antibody.

**Lentivirus construction and cell infection**

Plasmid encoding IκSR provided by Dr F Porteu (Institut Cochin, Paris) was inserted in a TRIP ΔU3-EF1α defective lentivirus expressing the green fluorescent protein (GFP) under an IRES. CD34⁺ cells were infected twice at day 0 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 CD34⁺ progenitors using the DNA Extraction kit BACC2 (GE Healthcare, Buckinghamshire, UK), treated in liquid phase by sodium bisulfite using Methyl Detector (Active Motif, Carlsbad, CA), dried, solubilized in RNAse-free water, and stored at -80°C until use. CD34⁺ cells embedded in agarose beads were treated with 0.2mg/mL proteinase K RNA grade (Invitrogen, Carlsbad, CA) overnight at 37°C in 10mM Tris-HCl pH 7.5, 10mM ethylenediaminetetraacetic acid (EDTA), 50mM NaCl, 0.2% sodium dodecyl sulfate (SDS). Bead-coated DNA was denatured by heating and incubated for 4h at 37°C in a fresh solution of 2.8M sodium bisulfite, 0.5mM hydroquinone and 0.6M NaOH, pH 5.8. Beads transferred to a Multiscreen®-HV (Millipore, Bedford,
MA) filtration plate were washed 8 times in 10mM Tris-HCl pH 8.0, 10mM EDTA and the reaction was stopped by 0.2N 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 Table S3. PCR products were purified using the QiAquick PCR Purification kit. For BM CD34+ 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, Foster City, CA).

**Chromatin immunoprecipitation**

HL-60, SW-480 cells or BM mononuclear cells were cross-linked with 1% formaldehyde for 10 min at room temperature, lysed in 50 mM Tris-HCl, pH 8.0 containing 1% sodium dodecylsulfate (SDS), 10 mM EDTA, and sonicated with a VibraCell Sonifier (Bioblock Scientific, Strasbourg, France). Chromatin immunoprecipitation (ChIP) experiments were performed on solubilized chromatin extracted from 10x10^6 cells and diluted 10-fold in ChIP dilution buffer (#20-153, Millipore). Chromatin was precleared for 1h and incubated overnight with 2µg 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 (Cambridge, UK) or 2µg p65/RelA antibody or isotypic IgG1-mouse/rabbit (Santa cruz Biotechnologies, Santa Cruz, CA). Immune complexes were collected on salmon sperm DNA/protein A agarose beads and washed. Modified histone/DNA complexes were eluted with 500µL 1% SDS, 0.1M NaHCO_3. After crosslink reversal, DNA was purified by phenol extraction. An aliquot of the sonicated chromatin was treated identically for use as input. qPCR for FAS, NFkBIA, B2M (Table S3) was performed with the following thermal cycling program: 5min at 95°C, 40 cycles of 30sec at 95°C, 30sec at 60°C, and 45sec at 72°C, followed by a 5-min extension time at 72°C. Relative enrichment of specific gene segment in ChIP was normalized to DNA input and control IgG and expressed as followed: 2^(Cq input-Cq gene) / 2^(Cq input-Cq IgG).

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

Reporter construction pGL2 FAS-Luc containing the -1519 to + 201 FAS promoter region spanning over the TSS (+1) was a gift from Dr LB Owen-Schaub (MD Anderson, Houston, TX) (19). Methylated plasmids were obtained by incubation of 5µg of pGL2 FAS-Luc or empty pGL2 with CpG Methyltransferase SssI (New England Biolabs, Ipswich, MA) during 4h at 37°C. DNA methylation was checked by digestion with the methylation sensitive
restriction endonuclease *HpaII*. Methylated or unmethylated plasmids were co-transfected in 293T cells \( (5 \times 10^4) \) at 300 ng/well with pRL-tk Renilla vector at 10ng/well to monitor transfection efficiency. At day 2 post-transfection, cells were lysed in Passive Lysis Buffer (Promega, Madison, WI) 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 NFκB inhibitor, BAY11-7082, inhibits Fas expression in HL60 cell line

To explore the role of NFκB 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 HL-60 that expressed Fas, and the colon carcinoma cell line SW480 that did not express the receptor. TNFα (10 ng/mL) increased the expression of Fas at the surface of the HL60 cells after 6 h, while it failed to induce Fas on SW480 cells. However, TNFα (2 h) stimulated the expression of another NFκB target, BclxL in these cells (Figure 1A). Consistently, TNFα upregulated the expression of FAS transcript in HL60, but not in SW480 cells, and BCL2L1 which encodes BclxL was upregulated after 2 h of incubation with TNFα in SW480 cells (Figure 1A). p65/RelA subcellular localization was analyzed by immunofluorescence in both cell lines at baseline and after 30 min of incubation with 10 ng/mL of TNFα. In HL60 cells, the p65/RelA protein was detectable in both the nucleus and the cytosol at baseline and after treatment with TNFα (Figure 1B). p65/RelA poorly present in the nucleus of SW480 cells, strongly relocalized to the nucleus after a 30-min treatment with TNFα and remained in the nucleus until 6 h (data not shown). These data show that Fas remained unexpressed in SW480 cell line although the TNFα-induced nuclear localization of p65/RelA and BCL2L1 transcription suggesting that the NFκB pathway was activated.

We then abrogated NFκB 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 5µM BAY11-7082 (Figure 1C). Immunoblot experiments confirmed the diminution of p65/RelA in the nucleus of BAY11-7082 treated cells (Figure 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 (Figure 1E,
BAY11-7082 also reduced, although less efficiently, the expression of *BCL2L1* transcript (Figure 1E, left) and decreased the expression of Fas at the cell surface (Figure 1E, right).

**p65/RelA binds to FAS promoter**

The *FAS* gene promoter contains three GGGRNNYYCC putative NFκB sites with R=A/G and Y=T/C in the 5’ UTR region spreading between -899 and the transcription start site (TSS; +1) (Figure 2A) (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 HL-60 cells treated with 5μM BAY 11-7082 for 6h. p65/RelA was precipitated by using 30-pb biotinylated oligonucleotides whose sequence contained either one of the 3 putative NFκB binding sites of the *FAS* promoter, or a consensus tandem NFκB binding site as a positive control, or a consensus *GFI-1B* binding site as a negative control. As shown in Figure 2B, p65/RelA bound to each of the 3 putative NFκB binding sites on *FAS* promoter, which was prevented by BAY11-7082. Using chromatin immunoprecipitation (ChIP) assays (Figure 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 NFκB site, but not to *B2M* promoter that does not have any NFκB site, both in HL60 or SW480 cells (Figure 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 NFκB. 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) (Figure 3A). While only 2 out of 35 CpG (6%) were methylated in the Fas-positive HL60 cell line, 24/35 CpG (68%) located both 5’ upstream of and within the CpG island were methylated in the Fas-negative SW-480 cells. In addition, ChIP experiments identified one active mark H3K9/14Ac and none of the two repressive marks H3K27me3 and H3K9me2 in HL60 cell line while the repressive mark H3K9me2 was expressed in SW480 cell line (Figure 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 µM azacitidine induced the demethylation of 20/24 CpGs in the SW480 cells, while it did not influence the CpG methylation in HL60 cells (Figure 3A). In these conditions, azacitidine did not induce cell apoptosis or necrosis (not shown) but clearly stimulated the expression of Fas in SW-480 but not in HL60 cells (Figure 3C, left and middle panels). Azacitidine also increased the recruitment of p65/RelA at FAS promoter in SW-480 cell line while it did not modify p65/RelA binding in the HL60 cell line (Figure 3C, right panel). We also transfected 293T cells with a pGL2 construct containing the FAS promoter region (-1519 to + 201 over the TSS) in either a methylated or an unmethylated conformation, inserted upstream of the luciferase gene reporter (19). As shown in Figure 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. TNFα (10 ng/mL for 6h) further increased the promoter activity that was abrogated by the NFκB inhibitor, BAY11-7082. Altogether, NFκB-dependent FAS gene expression is epigenetically regulated.

NFκB controls Fas expression in MDS/sAML bone marrow mononuclear cells

The clinical relevance of these findings was investigated in primary bone marrow (BM) myeloid cells collected from 53 patients with a MDS (n=43) or a secondary AML (n=10) and 15 healthy controls. Karyotype was unavailable in 7 patients. According to IPSS, 27 MDS patients had a low risk (LR)-MDS and 11 had a high risk (HR)-MDS (Table S1). In agreement with our previous data (26), the median Fas protein expression expressed on the
CD45loCD34+ bone marrow cells lower in sAML compared to MDS (P<0.001; Table S1). Serial samples (n=22) at the time of diagnosis and at the time of progression or transformation were studied for the expression of Fas (Figure 4A). In 16 cases at diagnosis, the level of Fas protein was elevated mostly in patients with percentages of BM blasts lower than 10%. Among those cases, 14 had a significant reduction of Fas level at the time of disease progression to high risk MDS or sAML, except for cases 14 and 28. The Fas RFI was inversely correlated with the percentage of BM blasts.

We then investigated the effect of TNFα on Fas expression. Primary BM mononuclear cells isolated from 4 Fas-negative samples of AML were incubated with 10 ng/mL TNFα for 6 h. Immuno-localization of p65/RelA was performed and demonstrated the presence of p65/RelA in the nucleus of blast cells at baseline. After treatment with TNFα, we observed an increment of the nuclear expression of p65/RelA in 3 cases (#3, 17, 49), suggesting that NFκB pathway could be activated in these cells (Figure 4B). However, Fas expression remained low after treatment with TNFα in the 4 cases both at transcript and protein levels, while BclxL expression increased in the same 3 cases (Figure 4B). This suggests that TNFα-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 NFκB in the regulation of FAS gene transcription, we treated BM mononuclear cells from patients with low/int-1 MDS (n=11) or high risk MDS/AML (n=10) with BAY11-7082 for 6h. Sixteen samples were positive for Fas before treatment and BAY11-7082 decreased the expression of Fas in 12 of them (Figure 5A).
We analyzed the subcellular localization of p65/RelA before and after BAY11-7082 treatment in 4 LR-MDS (2 RA, 1 RCMD-RS, 1, RAEB1) and 2 HR-MDS (1 sAML, 1 RAEB1 with a complex karyotype). **Figure 5B** shows two 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 BM cells (see 3 of the tested cases on **Figure 5C, upper panel**). In one case (#11), the sample size allowed following the kinetic of *FAS* and *BCL2L1* gene repression (**Figure 5C, lower panel**). In addition, lentivirus-mediated expression of an IκBα super-repressor (IκSR) 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 (**Figure 5D**). This suggests that NFκB may participate to the regulation of the transcription of *FAS* gene in primary BM 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* promoter in 4 Fas-expressing (3 RAEB1, 1 RAEB2) and 6 Fas-negative (1 RA, 1 RCMD-RS, 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 positive in all 4 Fas-positive low risk 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 high risk 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 (**Figure 6A**, $R^2 = 0.765$).

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 MDS/AML.
patients treated with azacitidine (26). Here, BM mononuclear or CD34+ cells from sAML patients (n=11) were cultured in vitro in the presence of TPO, IL-6, SCF and FLT3-L for 72 hours with or without 1µM azacitidine. In these conditions, cells did not differentiate nor they died (not shown). Azacitidine efficiently rescued Fas receptor expression on BM CD45lo/CD34+ blast cells in the 7 Fas-negative cases, while the effect was weak in two Fas-positive cases (#14 and #47) and null in another Fas-positive case (#13) (Figure 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 two patients. As shown in a representative experiment, azacitidine induced the demethylation of 14/16 CpG, including those located within NFκB binding sites 1 and 3 (Figure 6C). Second, we tested the effect of azacitidine on the binding of p65/RelA to FAS promoter in one case of sAML (#23) 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 NFκB transcription factor at FAS promoter in primary BM mononuclear cells (Figure 6D). Altogether, azacitidine can induce FAS promoter demethylation, thus facilitates p65/RelA binding and FAS gene transcription in myeloid cells.

Discussion

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

NFκB can promote cell survival through the transcriptional regulation of IAP (*X-IAP, cIAP1* and *cIAP2*) or BclxL (*BCL2L1*) family members or cell proliferation through the up-regulation of *CYCD1* and *c-MYC* (16, 27). NFκB can also facilitate cell death through the death receptor-mediated extrinsic pathway (19, 20, 27). These dual pro- and anti-apoptotic functions of NFκB could apply to MDS. In low risk MDS, the NFκB activity, that could be due to TNFα secretion (28-31), may account for *FAS* gene expression. NFκB pathway is constitutively activated when the disease progresses (12-16) and could suppress cell death through the transcriptional regulation of anti-apoptotic genes. In addition, the methylation of promoters could turn off the NFκB-dependent transcription of pro-apoptotic genes like *FAS* (32).

The present study demonstrates the direct up-regulation of *FAS* gene expression by NFκB in primary bone marrow cells. p65/RelA binds to the *FAS* gene promoter whereas NFκB inhibition with either BAY11-7082 (33) or the IκB super-repressor, IκSR, decreases Fas expression. The *FAS* gene has been shown previously to be a direct target for NFκB in lymphocytes, hepatocytes (6,20), and various solid tumor cell types (11, 32). The cell context may be essential as NFκB activation through the alternate pathway could be a *FAS* transcription repressor in MEL cells (34). Alternatively, the threshold of NFκB activity, which is under control of either the quantity of dimers p65/p65, p50/p50 and p65/p50 recruited to the chromatin, or post-translational modifications, or interactions with other transcription factors like Sp1 could determine its effects (35,36). For instance, while a high rate of activation could permit the transcription of a large spectrum of pro and anti-apoptotic genes, low levels of activation could restrict the transcription to few pro-apoptotic genes. In
our model of myeloid leukemogenesis, MDS at the beginning are characterized by inappropriate and excessive apoptosis linked to the upregulation of \textit{FAS} gene expression. At this stage, NF\textsubscript{x}B activation is weak while it increases as the disease progresses to AML together with the upregulation of anti-apoptotic genes like cIAPs, Bcl2 or downregulation of pro-apoptotic gene like FADD (16, 21, 37). In addition, the NF\textsubscript{x}B 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\textsubscript{x}B pathway could be crucial for cell fate decision between survival and death. Lastly, the cell fate could depend on the epigenetic state of the chromatin at NF\textsubscript{x}B target gene promoters. Our previous and current data support this later hypothesis as \textit{FAS} gene expression decreases along the progression of MDS to AML according to the increase of DNA methylation at \textit{FAS} gene promoter (26). The \textit{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 \textit{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\textsubscript{x}B 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 to high grade MDS in which the level of DNA methylation is high. Such an epigenetic regulation of NF\textsubscript{x}B-dependent transcription has been reported for \textit{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 MDS/AML patients could be used as a biomarker of the drug activity (26). It will be useful to determine whether this re-expression of Fas contributes to slow down the progression of the disease or constitutes a potential risk of treatment failure in a prospective clinical trial.

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References

1. Lavrik IN, Krammer PH. Regulation of CD95/Fas at the DISC. Cell Death Diff 2012 Jan;19(1):36-41.


31. Maciejewski J, Selleri C, Anderson S, Young NS. Fas antigen expression on CD34+ human marrow cells is induced by interferon gamma and tumor necrosis factor alpha and


Legend Figures

Figure 1: p65/RelA inhibitor, BAY11-7082, inhibits Fas expression in HL60 cell line

A. Kinetic of Fas expression upon TNF-α (10ng/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. Results are expressed as a ratio of median fluorescence intensity (RFI) to isotypic control or normalized relative quantities (NRQ) of FAS or BCL2L1 to GAPDH, RNA18S and HPRT, respectively. B. Immuno-localization of p65/RelA before and after 30 min of treatment with 10 ng/mL TNFα in HL60 and SW480 cell lines. Nuclei were colored with DAPI. C. Immuno-localization of p65/RelA in HL60 cells untreated (0) or treated with 5µM of BAY11-7082 for 2, 4 or 6 h. Nuclei were colored with DAPI. B. One representative of three independent experiments. D. Western blot for p65/RelA protein in nuclear and cytoplasmic extracts prepared from HL60 cell line, treated or not with 5µM of BAY11-7082 for 6 h. Hsc70 is a loading control. E. Kinetic of Fas expression upon treatment with 5µM of BAY11-7082 in HL60 cell line by quantitative real time PCR or flow cytometry. Results are expressed as NRQ normalized relative quantities using the ΔCq method or as RFI, respectively. BCL2L1 gene was used as a positive control.

Figure 2: p65/RelA binds to FAS promoter

A. Schematic representation of 5'UTR of the FAS gene. Putative NFκB consensus binding sites are indicated upstream of transcription start site (TSS). Arrows indicate the position of primers for chromatin immuno-precipitation of p65/RelA.

B. In vitro DNA-binding affinity precipitation experiment of p65/RelA. HL-60 nuclear extracts were incubated with biotinylated-oligonucleotides deriving from each of the putative
NFκB binding sites at FAS promoter (Fas1, Fas2, Fas3), or a tandemly repeated consensus sequence for NFκB as a positive control, or an oligonucleotide deriving from the promoter of GFI1B gene as a negative control. Oligoprecipitates (OP) were trapped on streptavidin beads and harvested by centrifugation. OP and the corresponding supernatants (SPN) were analyzed on two separated gels by Western blotting using antibodies to p65/RelA. One representative of three independent experiments.

C. Chromatin immunoprecipitation of p65/RelA at FAS gene promoter in SW480 cell line or HL-60 cell line treated or not with 10ng/mL TNF-α for 6 hours. NFKBIA and B2M gene were amplified as positive and negative controls, respectively. Enrichment of p65/RelA compared to DNA input and control isotypic IgG (means ± SD of 2 independent experiments).

**Figure 3: DNA methylation level at FAS promoter regulates the binding of p65/RelA to the chromatin.**

A. DNA methylation of CpG dinucleotides at FAS gene promoter analyzed on bisulfite-treated genomic DNA by PCR and sequencing. Upper panel: Schematic representation of the CpG dinucleotides (vertical lines) and CpG island in the 5'flanking region of FAS gene. Lower panel: CpG methylation before and after treatment with 1 µM azacitidine for 3 days in HL60 and SW480 cell lines. Filled circles: fully methylated CpGs; open circles: unmethylated CpGs.

B. Chromatin immunoprecipitation assay of histone marks at FAS, B2M, RAG1 and PAX6 genes in HL60 (white bars) and SW480 (grey bars) cell lines. Enrichment of H3K9/14ac and H3K4me2 active marks or H3K27me3 and H3K9me2 repressive marks to DNA input and control isotypic IgG are means ± SD of three independent experiments.
C. Effect of azacitidine (1 µM for 3 days) on Fas expression by flow cytometry as RFI (left panel), FAS transcript level by RT-qPCR as normalized relative quantity (NRQ) to GAPDH (middle panel) and ChIP for p65/RelA at FAS promoter (right panel) in HL-60 (white bars) and SW-480 (grey bars) cell lines. Student t-test for P values (* P<0.05 and ** P<0.01).

D. Luciferase assay for in vitro measurement of FAS promoter activity. 293T cells co-transfected with methylated or unmethylated Fas plasmids and pRL-tk Tenilla vector were treated with 10 ng/ml TNF-α or 5 µM BAY11-7082 for 6h. Normalized reporter activity is expressed as the firefly luciferase value to the Renilla luciferase value for each experimental condition. Mean ± SD of 3 independent experiments.

Figure 4: Fas expression on CD45loCD34+ MDS/AML cells is inversely correlated to the BM blast count and remains low in AML cells after treatment with TNFα

A. Upper panel: Flow cytometry analysis of membrane Fas expression on CD45loCD34+ bone marrow cells in 22 MDS patients (19 LR-MDS and 3 HR-MDS) at diagnosis (white bars) and at the time of progression (black bars). Results are expressed as ratio of median fluorescence intensity (RFI) to isotypic control. Middle panel: Bone marrow blast percentages. Right panel: Inverse correlation between Fas expression and % of bone marrow blasts. B. AML blasts (n=4) were treated for 6 h with TNFα (10 ng/mL) before study of p65/RelA subcellular localization by immunofluorescence, quantification of FAS or BCL2L1 transcripts by RT-qPCR, without TNFα (white bars), with TNFα (hatched bars), and Fas or BclxL proteins measurement by flow cytometry. Results are expressed as normalized relative quantities (NRQ) or as ratio of fluorescence intensities (RFI), respectively.
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 CD45loCD34+ cells before and after treatment with 5µM BAY11-7082 for 6 hours. Results are expressed as a ratio of median fluorescence intensity (RFI) to isotypic control. B. Inhibition of p65/RelA nuclear localization by BAY11-7082 (5 µM for 6h) in MDS/AML (n=6) mononuclear cells. Representative fluorescence microphotographs of p65/RelA labeling in one low risk MDS (LR-MDS) and one secondary AML (sAML). Nuclei are colored with DAPI. C. RT-qPCR for FAS and BCL2L1 gene expression in MDS mononuclear cells treated or not with 5µM of BAY11-7082 expressed as normalized relative quantity (NRQ) to GAPDH and B2M according to the ΔC_q method. D. NFκB pathway inhibition by lentiviral expression of IκSR 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 panel) and Fas expression by flow cytometry (right panel). Results representative of three independent experiments

Figure 6: In vitro re-induction of FAS gene expression with azacitidine in MDS/AML.

A. Upper panel: Chromatin immunoprecipitation of p65/RelA at the FAS promoter in 10 MDS or sAML samples. Enrichment of p65/RelA compared to DNA input and control isotypic IgG. Middle panel: Flow cytometry analysis of Fas receptor on bone marrow CD45loCD34+ progenitors. Results are expressed as ratio of median fluorescence (RFI) to isotypic control. Dotted line indicates the threshold of positivity for Fas expression in primary bone marrow samples. Bottom panel: Correlation with Fas expression by flow cytometry.

B. Flow cytometry for Fas expression on bone marrow CD45loCD34+ progenitors before (white bars) and after (black bars) in vitro treatment with 1 µM azacitidine for 3 days in 11
MDS/sAML samples. Results are expressed as a ratio of median fluorescence intensity (RFI) to isotypic control.

C. CpG methylation analysis. MDS/sAML CD34⁺ cells (n=2) were treated for 3 days with 1 µM azacitidine. Fas promoter was amplified on bisulfite-treated DNA from CD34⁺ cells. Amplicons were cloned in bacteria and sequenced (10 clones per sample). Black circles: fully methylated CpGs; open circles: unmethylated CpGs.

D. Chromatin immunoprecipitation of p65/RelA at FAS promoter (n=1). Enrichment of p65/RelA compared to DNA input and control isotypic IgG.
Figure 1: p65/RelA inhibitor, BAY11-7082 inhibits Fas expression in HL-60 cell line

A

B

C

D

E

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Figure 2: p65/RelA binds to FAS promoter

A

NFκB binding sites

B

Consensus  Fac1  Fac2  Fac3 facet

Bay11-7082

OP  SPN

C

Relative expression

FAS  WRF44  EWM
Figure 3: DNA methylation level at FAS gene promoter regulates the binding of p65/RelA to the chromatin.

A

B

C

D

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Figure 4: Fas expression on CD45<sup>hi</sup>CD34<sup>+</sup> progenitors is inversely correlated to the bone marrow blast count.
Figure 5: BAY11-7082 decreases Fas expression in MDS/AML bone marrow mononuclear cells

A

B

C

D
Figure 6: Fas expression correlates with p65/RelA binding to the chromatin in MDS/AML mononuclear cells

In vitro induction of FAS gene expression with azacitidine
Molecular Cancer Research

Epigenetic control of NFκB dependent FAS gene transcription during progression of myelodysplastic syndromes

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