Elevated levels of STAT1 in Fanconi anemia group A lymphoblasts correlate with the cells' sensitivity to DNA interstrand crosslinking drugs

Inés Prieto-Remón, Dámaso Sánchez-Carrera, Mónica López-Duarte, Carlos Richard, and Carlos Pipaón

Servicio de Hematología, Instituto de Formación e Investigación Marqués de Valdecilla-IFIMAV, Santander, Spain

ABSTRACT

Progressive bone marrow failure starting in the first decade of life is one of the main characteristics of Fanconi anemia. Along with the bone marrow failure, this pathology is characterized by congenital malformations, endocrine dysfunction and an extraordinary predisposition to develop cancer. The fact that hematopoietic progenitor cells from subjects with Fanconi anemia are sensitive to both DNA-interstrand crosslinking agents and inflammatory cytokines, which are aberrantly overproduced in these patients, has led to different explanations for the causes of the bone marrow failure. We analyzed STAT1 expression in lymphoblastoid cell lines derived from patients with Fanconi anemia group A and correlated this with aspects of the Fanconi anemia phenotype such as sensitivity to genotoxic agents or to inhibitory cytokines. We provide evidence of overexpression of STAT1 in FANCA-deficient cells which has both transcriptional and post-translational components, and is related to the constitutive activation of ERK in Fanconi anemia group A cells, since it can be reverted by treatment with U0126. STAT1 phosphorylation was not defective in the lymphoblasts, so these cells accumulated higher levels of active STAT1 in response to interferon gamma, probably in relation to their greater sensitivity to this cytokine. On the other hand, inhibition of STAT1 by genetic or chemical means reverted the hypersensitivity of Fanconi anemia group A lymphoblasts to DNA interstrand crosslinking agents. Our data provide an explanation for the mixed sensitivity of Fanconi anemia group A cells to both genotoxic stress and inflammatory cytokines and indicate new targets for the treatment of bone marrow failure in these patients.

Introduction

Fanconi anemia (FA) is a recessive congenital disorder clinically characterized by bone marrow failure, congenital malformations, endocrine dysfunction and an extraordinarily marked predisposition to neoplasms. Patients develop a progressive thrombocytopenia and eventually pancytopenia during the first decade of their life which can be treated with androgens but in most cases requires hematopoietic stem cell transplantation under specific protocols adapted to the patients' characteristics. Although great advances have been achieved, the drugs used sometimes have undesirable side effects, and is not always possible to find a related donor for the transplant, leading to severe complications. A better understanding of the molecular basis of bone marrow failure in these patients could help in the development of new treatments.

To date, 15 different genes have been involved in the development of FA; these are designated as *FANC* genes. The assimilation of some of the *FANC* gene products with previously known proteins involved in DNA repair pathways led to the discovery of their participation in nuclear complexes involved in the resolution of DNA interstrand crosslinks generated during the normal physiology of the cell or induced by certain drugs.[1] In addition to their participation in the FA-BRCA nuclear DNA repair complex, certain FANC proteins are involved in signaling pathways, which has shed light on other aspects of the FA phenotype, such as the endocrine dysfunction and the sensitivity to inflammatory cytokines.[2,3] Moreover, the combination of the sensitivity of FA progenitor hematopoietic cells to inflammatory cytokines with the elevated production of interferon-gamma (IFNγ), tumor necrosis factor alpha (TNFα) and interleukin-1beta (IL-1β) in these patients is thought to contribute to their bone marrow failure and posterior malignization.[4-6] Thus, two different mechanisms are nowadays considered to participate in the bone marrow failure of FA patients: on the one hand, FA cells accumulate DNA damage in each division, which results in exacerbated p53 activation and apoptosis of their hematopoietic progenitors[7] and, on the other hand, FA patients overproduce inhibitory cytokines, which eventually degrade their bone marrow.[8]

Signal transducers and activators of transcription (STAT) are transcription factors activated by phosphorylation triggered by ligand-bound cell surface receptors. STAT factors mediate signaling initiated by many extracellular stimuli including cytokines and growth factors and induce responses such as cell proliferation, differentiation and apoptosis.[9] STAT1 is phosphorylated at tyrosine 701 in response to IFNγ activation and is then translocated to the nucleus where it promotes the transcription of specific genes. Interestingly, FANCC was demonstrated to interact with and be necessary for the correct activation of STAT1 in response to IFNγ.[10] The importance of STAT1 in IFNγ signaling was demonstrated in studies of mutant cell lines and mice. STAT1 knockout mice show high susceptibility to microbial and viral infections as well as tumor formation due to the abrogation of induction of certain target genes.[11,12] Recently, STAT1 has also been impli-

 doi:10.3324/haematol.2012.074187
Manuscript received on July 12, 2012. Manuscript accepted on December 17, 2012.
Correspondence: cpipaon@humv.es

cated in the induction of apoptosis in response to DNA damage,[13-15] a process regulated by its interaction with p53.[16]

In this study we investigated STAT1 expression in FANCA-deficient cells and its role in the sensitivity of FA-A lymphoblastoid cell lines (LCL) to DNA interstrand crosslinking agents.

Design and Methods

Cell culture and cell viability assay

Using a Ficoll gradient method, peripheral blood mononuclear cells were separated from peripheral blood samples from healthy donors and FA patients after their informed consent had been obtained. Epstein-Barr virus (EBV)–transformed human lymphoblasts derived from FA-A or FA-C patients and their phenotypically corrected counterparts were maintained in RPMI 1640 medium (Life Technologies, Rockville, USA) supplemented with 10% heat-inactivated fetal calf serum and grown in a humidified 5% CO_2-containing atmosphere at 37°C. Phenotypically-corrected FA LCL either harbor a reversion of the original FANCA mutation (rFA-A), or were transduced with a wild type FANCA cDNA using a viral vector (FA-A+A). Sensitivity to DNA interstrand crosslinking agents was tested to ascertain their phenotypic correction. Cells were grown in the presence of IFNγ (Applichem, Darmstadt, Germany), TNFα (Sigma, St. Louis, USA), U0126 (Merck KGaA, Darmstadt, Alemania), SB203580 (Cayman, Ann Arbor, USA) or epigallocatechin-3-gallate (EGCG) (Cayman, Ann Arbor, USA) where indicated. To determine the cell resistance to certain treatments, 50,000 cells were plated in 96-well plates and incubated for 3 days in the presence of the indicated drugs. Cell viability was then determined using the XTT Cell Proliferation Kit II (Roche, Basel, Switzerland) following the manufacturer's instructions. Human bone marrow and cord blood were collected under approval of the Cantabrian Research Ethics Committee.

Reverse transcriptase polymerase chain reaction analysis

Total RNA was prepared using TRI reagent (MRC, Cincinnati, USA). To assess mRNA expression, both quantitative and semi-quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) methods were used. For the reverse transcriptase reaction, RNA (5 μg) was primed with random hexamers and reverse transcribed with Superscript MMLV reverse transcriptase (Invitrogen, Carlsbad, USA) in a 20 μL volume following the manufacturer's instructions. The cDNA generated was amplified using primers for human STAT1 (5'-AACAGAAAAATGCTGGCACC and 5'-AGAGGTCGTCTCGAGGTCAA), STAT1α/β (5'-ACGGAGGCGAACCTGACTTCCA and 5'-AGCCCACTATCCGAGACACCTCG or 5'-GTCACTCTTCTGTGTTCACTTAC), STAT3 (5'-GCCATCTTGAGCACTAAGCC and 5'-GCTACCTGGGTCAGCTTCAG), STAT5a (5'-GGAGAATTCGACCTGGATGA and 5'-TGGATGCCATGATCTGAAAA), STAT5b (5'-CTATGCCACACAGCTCCAGA and 5'-CCTCCAGAGACACCTGCTTC), PIAS1 (5'-GCAGCCTTCCAGGTTACCTTCCA and 5'-AGGGCCCGACACGGAATTGT), SOCS5 (5'-CAGGAACAAGACTTGCACGA and 5'-GGAAAACACAAGCCCACAGT), CISH (5'-GCCAGAAGGCACGTTCTTAG and 5'-GTACAAAGGGCTGCACCAGT), IFI16 (5'-GCTGACCGAAACATGGAGAT and 5'-CAGATCTCAACTCCCCGGTA), MX1 (5'-TGTGCAGCCAGTATGAGGAG and 5'-CCCACAGCCACTCTGGTTAT), ICAM1 (5'-GGCTGGAGCTGTTTGAGAAC and 5'-ACTGTGGGGTTCAACCTCTG), IFRD1 (5'-TGCCCAATCAATGAAGTGAA and 5'-TCTGTTGGAAAATCCCGTTC), p21 (5'-GAGGCACTCAGAGGAGGCGCCAT and 5'-CACACGCTCCCAGGCGAAGTC) and β2 microglobulin (β2μ) (5'-GAGACATGTAAGCAGCATCA and 5'-AGCAACCTGCTCAGATACAT). After 30 amplification cycles, except for β2μ (25 cycles), the expected PCR products were size-fractionated on a 2% agarose gel and stained with ethidium bromide. For the quantitative analysis, expression values of triplicate measurements were normalized against a housekeeping gene.

RNA Interference assays

To genetically block the expression of STAT1 in LCL, we transduced a shRNA construct against STAT1 (reference TRCN0000280024 from Sigma, St. Louis, USA) into the cells and selected the transduced cells by cell sorting. The efficacy of the STAT1 blockade was tested by analyzing its mRNA expression according to the manufacturer's instructions.

Western blot analysis

To prepare whole cell lysates, cells were collected by centrifugation and washed once with phosphate-buffered saline and then lysed in EBC buffer [50 mM Tris (pH 8.0), 120 mM NaCl, 0.5% Nonidet P-40] supplemented with aprotinin (11.5 μg/mL), leupeptin (11.5 μg/mL) phenylmethylsulfonyl fluoride (50 μg/mL), sodium fluoride (100 mM) and sodium orthovanadate (0.2 mM). Protein concentration was determined by the bicinchoninic acid (BCA) method according to the manufacturer's instructions (Pierce, Rockford, USA). Proteins (100 μg) were resolved in sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride filters. Blots were incubated with rabbit antibodies against P-Tyr-STAT1, P-ERK, ERK2, PARP or GAPDH (Santa Cruz, Santa Cruz, USA) and then incubated with goat anti-rabbit or anti-mouse antibodies conjugated with horseradish peroxidase (Pierce, Rockford, USA). Bound antibody was detected by a chemiluminescence assay (Pierce, Rockford, USA).

Gene reporter assay

A genomic PCR fragment of 628 base pairs (bp) (-588 to +40, referred to the initiation of transcription) from the 5' regulatory region of STAT1 was amplified using specific primers and cloned into the pGL2basic luciferase reporter vector (Promega, Madison, USA). 293T cells were co-transfected with 2 μg pGL2–STAT1-588/+40 construct, 1 μg of an expression vector for hRasV12-S35 and 0.2 μg pRSV–β-gal in triplicate using Superfect (Qiagen, Düsseldorf, Germany) according to the manufacturer's directions. Thirty-six hours after transfection, cell extracts were prepared and analyzed for their luciferase activity by using a reporter gene assay system (Promega, Madison, USA). Results were normalized for transfection efficiency with the β-galactosidase activity values obtained in the same extracts.

Results

STAT1 is overexpressed in Fanconi anemia group A lymphoblastoid cell lines

Our previous results led us to test the functionality of the STAT1 pathway in FA-A LCL. Data from Pang *et al.* demonstrated an altered function of the JAK-STAT pathway in FANCC-deficient cells.[10,17] Surprisingly, we discovered that the STAT1 protein was overexpressed in LCL derived from FA-A patients compared to the levels in cell lines derived from healthy donors (Figure 1A). In order to confirm that STAT1 overproduction was only due to the lack of functional FANCA protein in these cells, we trans-

duced a wild-type FANCA cDNA into FA-A LCL which corrected their phenotype. Using western blotting, we were able to confirm a reduction in the levels of STAT1 protein in FA-A corrected cells of two different cell lines (Figure 1B), demonstrating the involvement of FANCA in the expression of this protein. Interestingly, we observed no variation in the level of STAT1 protein in FANCC-deficient cells compared to their isogenic corrected counterparts (Figure 1C), suggesting a specific role of FANCA protein in the modulation of STAT1 expression. We next wanted to confirm that the results obtained in our LCL are an effect of what happens in the patients and not a consequence of the immortalization process. To this end, we analyzed the amount of STAT1 protein in primary samples of bone marrow: STAT1 protein accumulated to a higher level in the samples analyzed (Figure 1D).

We also investigated whether the accumulation of the STAT1 transcription factor is paralleled by an accumulation of its messenger RNA. STAT1 mRNA expression was roughly 2-fold higher in FA-A LCL than in LCL derived from healthy donors (Figure 1E). To verify the involvement of FANCA dysfunction in this finding, we analyzed two different FA-A LCL and their respective phenotypically corrected counterparts by quantitative RT-PCR. We observed a 2-fold increase in STAT1 mRNA accumulation in FA-A LCL (Figure 1G). No increase, or even a decrease, was observed in FANCC-deficient cells compared to their FANCC-transduced counterparts (Figure 1I). We also analyzed the expression of the mRNA coding for the α and β isoforms of STAT1. The mRNA for both isoforms was overexpressed in FANCA-deficient LCL compared to either LCL derived from healthy subjects or to the isogenic corrected cell lines transduced with a wild-type FANCA cDNA (Figure 1E,H). Other members of the STAT family showed no basal overexpression in FANCA-deficient cells when compared to their FANCA-transduced counterparts (Figure 1H). Finally, we analyzed the expression of STAT1 mRNA in primary mononuclear cells from peripheral blood samples of FA-A patients. Overall, STAT1 mRNA expression was higher in samples from patients than in samples obtained from healthy donors (Figure 1F).

All these data demonstrate that the lack of a functional FANCA protein leads to overexpression of STAT1 protein in FA patients.

STAT1 protein accumulation in Fanconi anemia group A lymphoblastoid cell lines depends on transcriptional and post-translational mechanisms

In order to discard a putative mRNA stabilization mechanism in the accumulation of STAT1 mRNA in FA-A cells, we studied its degradation after blocking general transcription with actinomycin D. STAT1 mRNA has a half life of about 12 h in LCL, but the profile of its decay was similar in FA-A and control cells (Figure 2A), suggesting that STAT1 mRNA overexpression has a transcriptional basis in FANCA-deficient cells. However, the small differences at the mRNA level do not correlate perfectly with the strong induction of the STAT1 protein in FA-A LCL (compare Figures 1A and 1E), so we suspected that post-translational mechanisms could be contributing to STAT1 protein accumulation. To support this idea, we studied STAT1 protein half-life in FA-A LCL. After cycloheximide treatment, the level of STAT1 protein remained steady for about 3 h in FANCA-proficient cells, with a profound decay between 3 and 17 h (Figure 2B). In contrast, in FA-A LCL, the levels of STAT1 protein remained high for up to 17 h after cycloheximide treatment, indicating an alteration in the mechanism of renewal of this protein in FANCA-deficient cells. Collectively, these results demonstrate that FANCA deficiency leads to an increased transcription of the *STAT1* gene and a stabilization of the STAT1 factor.

FANCA-deficient cells phosphorylate STAT1 normally in response to interferon-γ

FANCC protein has been reported to be necessary for the correct activation of STAT1 in response to IFNγ.[10] We wanted to know if STAT1 is correctly activated in FANCA-deficient cells. We analyzed the accumulation of tyrosine 701-phosphorylated STAT1 in FA-A LCL after treatment with IFNγ by western blotting using a specific antibody. Our results showed that STAT1 is not only phosphorylated normally in FANCA-deficient cells but also that the levels of Tyr701-P-STAT1 rise much higher in response to IFNγ, probably due to the elevated levels of the STAT1 protein in these cells (Figure 3A). When we performed a time course of this phosphorylation in LCL, we observed a rapid increase in Tyr701-P-STAT1, which peaked at about 30 min and then returned to normal levels about 2 h after treatment. This profile of induction was essentially identical in FA-A LCL, although the increase in Tyr701-P-STAT1 was much greater (Figure 3B).

These data demonstrate that tyrosine phosphorylation of STAT1 in response to IFNγ is not defective in FANCA-deficient LCL and suggest that the high STAT1 levels in FANCA-deficient hematopoietic progenitor cells could generate an exaggerated response to the high levels of IFNγ produced by FA patients, contributing to these patients' bone marrow failure.

Lymphoblastoid cell lines do not respond to interferon-γ or tumor necrosis factor-α

It has been reported that the hypersensitivity of the FA hematopoietic stem cells to inhibitory cytokines such as IFNγ and TNFα, which are overproduced by FA patients, may be related to the progressive bone marrow failure.[4,18,19] The fact that STAT1 is phosphorylated in response to IFNγ demonstrates the presence of functional receptors for this cytokine in FA-A LCL (Figure 3A,B). Interestingly, although IFNγ induced the binding of STAT1 to a consensus binding site both in FA-A LCL and their corrected counterparts in an electrophoretic mobility shift assay, no difference between them was detected (*data not shown*). In an attempt to correlate the overexpression of STAT1 with a stronger response of FA-A hematopoietic progenitors to IFNγ, we used our LCL model to analyze the basal and IFNγ-induced expression of a group of genes known to respond to this cytokine. Thus, we treated FA-A LCL with increasing concentrations of IFNγ and studied their mRNA expression by quantitative RT-PCR. Some of the genes tested, such as *MX1* and *CISH*, showed higher basal expression in FA-A cells than in their corrected counterparts. Others showed very little or no differences in basal mRNA expression. IFNγ did not induce changes in the expression of any of these genes (Figure 3C). Despite of these results, we tested whether LCL could reproduce the sensitivity of FANCA-deficient hematopoietic progenitors to inhibitory cytokines. The viability of LCL did not change in response to increasing doses of IFNγ, TNFα or the combination of both, independently of their FANCA

status (Figure 3D).

Collectively, these result suggests that although STAT1 is normally phosphorylated in tyrosine 701, its function in IFNγ signaling is abrogated in LCL, probably as part of the immortalization process by Epstein-Barr virus.

STAT1 overexpression plays a role in the sensitivity of Fanconi anemia group A lymphoblastoid cell lines to DNA interstrand crosslinking agents

There is accumulating evidence that STAT1 plays a key role in various forms of cell death.[15] It is well known that FANCA-deficient cells are very sensitive to DNA crosslinking drugs. We used a XTT assay to confirm the decrease in cell viability of our FA LCL at doses of cisplatin lower than those used for their corrected counterparts (Figure 4A). In order to establish a role of STAT1 in this hypersensitivity, we interfered with STAT1 expression by transfecting a small-hairpin RNA (shRNA) specific for this gene into FA-A LCL. This construct was able to reduce STAT1 mRNA expression in about 50% (Figure

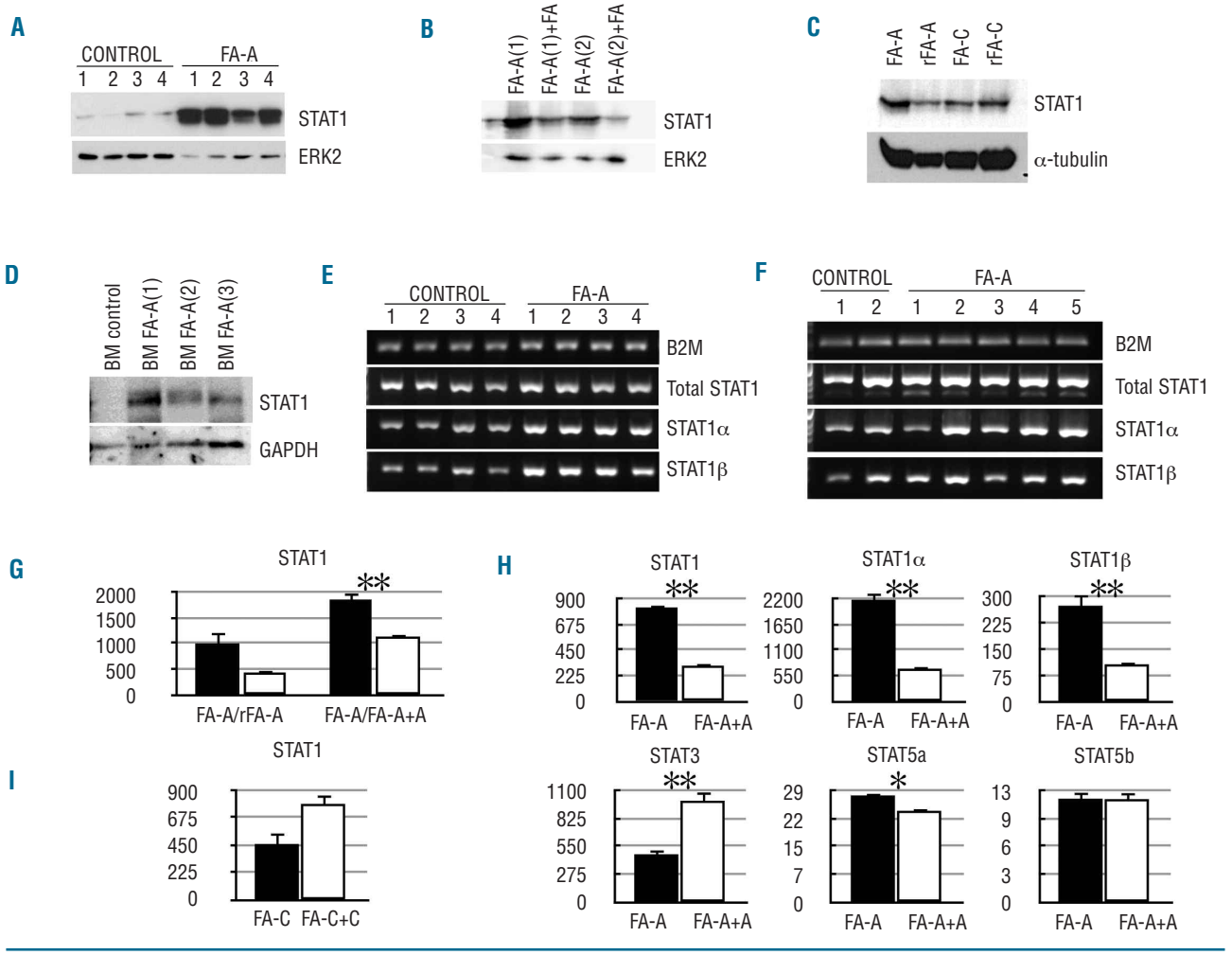

Figure 1. STAT1 is overexpressed in FA-A cells. (A) Whole cell protein extracts from LCL derived from FA-A patients or healthy donors were analyzed by western blot for the expression of STAT1. ERK2 expression is shown as a loading control. (B) Two different FA-A LCL (1 and 2) were phenotypically reverted by transduction of a wild-type FANCA cDNA (rFA-A). Expression of STAT1 in their protein extracts was studied by western blot. Similar levels of ERK2 demonstrate equal loading. (C) FA-A and FA-C LCL were transduced with FANCA and FANCC cDNA respectively, to correct their phenotype (rFA). Expression of STAT1 in the cells was analyzed by western blot of their protein extracts. Alfa tubulin expression is shown as a loading control. (D) Bone marrow samples from three different FA-A patients and a healthy donor were cleared of erythrocytes and their protein extracts analyzed by western blot. STAT1 was detected using a specific antibody and GAPDH protein levels are shown to assure equal loading. (E) Total RNA extracts from LCL derived from four different FA-A patients and four healthy donors were analyzed by semiquantitative RT-PCR for the mRNA expression of total STAT1 or its isoforms STAT1α and STAT1β. β2-microglobulin (B2M) expression is shown as a houskeeping control. (F) Semiquantitative RT-PCR analysis of total RNA extracts obtained from primary peripheral blood mononuclear samples from five FA-A patients and two healthy donors. Total STAT1, STAT1α and STAT1β mRNA expression is studied. B2M expression is shown as a houskeeping control. (G) STAT1 expression was analyzed in total RNA extracts from two different FA-A LCL (black bars) and their respective phenotypically corrected counterparts (white bars) by quantitative RT-PCR. The correction was carried out by two different methods: FA-A LCL on the left were corrected by the reversion of the original FANCA mutation (rFA-A), while FA-A LCL on the right were stably transduced with the wild-type FANCA cDNA using a viral vector (FA-A+A). The statistical significance of the data shown in panels (G), (H) and (I) was tested by an independent t test and the reported *P* values are two-tailed (**P*<0.05, ***P*<0.01). (H) Quantitative RT-PCR analysis of the expression of total STAT1, its α and β isoforms as well as other members of its protein family in total RNA extracts from FA-A LCL (black bars) and their FANCA-transduced counterparts (white bars). (I) STAT1 mRNA expression in total RNA extracts from FA-C LCL (black bars) and their FANCC-tranduced corrected counterparts (white bars).

4B). The sensitivity to cisplatin of these cells transduced with the construct normalized, as measured by the number of propidium iodide-positive cells under these conditions (Figure 4C), or by using a XTT viability assay (Figure 4D).

EGCG is a STAT1 inhibitor that is able to protect cells from apoptosis.[20,21] We decided to use this compound in our FA-A LCL to analyze its effect on the characteristic sensitivity of these cells to DNA interstrand crosslinking agents. First, we studied its effect on STAT1. We found that EGCG reverts STAT1 mRNA levels to those observed in corrected LCL (Figure 4E). This effect seems specific among other members of the STAT family (Figure 4E). When we treated FA-A LCL with EGCG and increasing doses of cisplatin to test its effect on their viability, cells became more resistant to cisplatin in an EGCG dose-dependent manner (Figure 4F).

These data demonstrate that STAT1 overexpression has a key role in the sensitivity of FA-A LCL to DNA interstrand crosslinking agents.

The activation of ERK in Fanconi anemia group A cells is responsible for the STAT1 accumulation in these cells

Several groups have reported a constitutive activation of ERK in FANCA-deficient cells.[22] In addition, we observed that the stimulation of FA-A LCL with mitomycin C induced a temporary increase in the phosphorylation of ERK, with this effect being stronger in FANCA-deficient cells (Figure 5A), suggesting a role of this kinase in the mitomycin C-sensitive phenotype of these cells as determined by the persistency of digested PARP in them. Since our data support a role of STAT1 in the sensitivity of FANCA-deficient cells to DNA interstrand crosslinking agents, we wondered whether the activation of ERK in FA-A cells might modulate the levels of STAT1. We treated FA-A LCL with increasing doses of U0126, an inhibitor of the activation of ERK, and observed a progressive reduction in the amount of both STAT1 factor and its messenger (Figure 5B,C). In addition, a region of the sequence upstream of the transcriptional start site of the gene was activated when co-

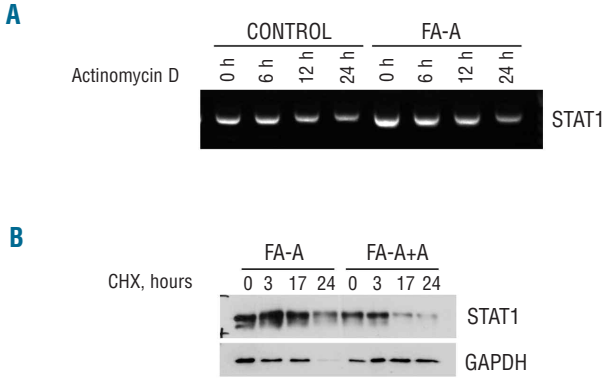

Figure 2. STAT1 overexpression in FA-A LCL depends on transcriptional and post-translational mechanisms. (A) LCL derived from a FA-A patient and a healthy donor were treated with actinomycin D for the indicated times. Total RNA was extracted at the different time points and the amount of STAT1 mRNA was determined by semiquantitative RT-PCR. (B) To study the stability of the STAT1 factor in FA-A LCL and their FANCA-transduced counterparts, the cells were treated with 30 μM cycloheximide (CHX) for the indicated times. Whole cell extracts from those time points were analyzed by western blot using anti-STAT1 or anti-GAPDH antibodies.

transfected with RasV12-S35, a mutant of Ras that specifically activates the Raf pathway (Figure 5D). These results demonstrate that the constitutive activation of ERK increases the expression of STAT1 in FA-A LCL.

We then investigated whether the reduction in STAT1 induced by the inhibition of ERK could affect the sensitivity of FA-A LCL to cisplatin, an ICL-generating drug. Treatment with U0126 improved the viability of FA-A LCL in response to cisplatin, in contrast with the treatment with SB203580, an inhibitor of p38, which showed no effect (Figure 5E). These data further confirm a key role of STAT1 in the sensitivity of FA-A LCL to ICL-generating agents and reveal ERK as a modulator of its expression in these cells.

Discussion

One of the most important clinical manifestations of FA is progressive bone marrow failure, which threatens the life of patients in the first decade of their life. The currently accepted idea is that the depletion of hematopoietic stem cells is a consequence of the failure of the DNA repair mechanisms in FA cells which exacerbates apoptosis.[7] However, FA cells are also prone to apoptosis induced by inflammatory cytokines, which are aberrantly overproduced in these patients.[6,18] There is now increasing evidence supporting the importance of these inhibitory cytokines in the bone marrow failure of FA patients. Indeed, a recent study was designed to find inhibitory molecules able to reduce the production of TNFα in FA patients and, therefore, retard the patients' progressive bone marrow failure.[8] In the present study, we found evidence of overexpression of STAT1 in FANCA-deficient cells and how this may explain the hypersensitivity of FA-A cells to both genotoxic agents and to inflammatory cytokines.

Most of the studies dealing with the sensitivity of FA cells to inflammatory cytokines have been carried out on FA-C LCL. This model allowed the discovery that the FANCC protein interacts directly with STAT1 facilitating the latter's activation in response to IFNγ.[10,23] Here, we report a greater accumulation of STAT1 and its messenger in FA-A LCL compared to LCL derived from healthy donors. Although we confirmed this observation in a group of LCL derived from different FA-A patients, in order to discard a possible differential influence of the immortalization process in STAT1 overexpression, as controls for the FA-A cell lines we used isogenic counterparts in which the FANCA gene was either spontaneously reverted or introduced in its wild-type form using a viral vector. Restoration of FANCA function, demonstrated by reversion of the hypersensitivity to interstrand DNA crosslinking agents (Figure 4A), reduced STAT1 protein and mRNA levels in FA-A LCL. We analyzed the stability of STAT1 mRNA and protein to gain some insight into the mechanism by which the lack of a functional FANCA protein leads to an induction of STAT1 expression. Our data suggest this mechanism is complex, involving regulation of STAT1 gene transcription and stabilization of the STAT1 protein. This agrees with data from other groups reporting both types of STAT1 regulation in acute myeloid leukemia cell lines.[24]

The overexpression of STAT1 in FA-A cells suggests it may contribute to the hypersensitivity of FA-A

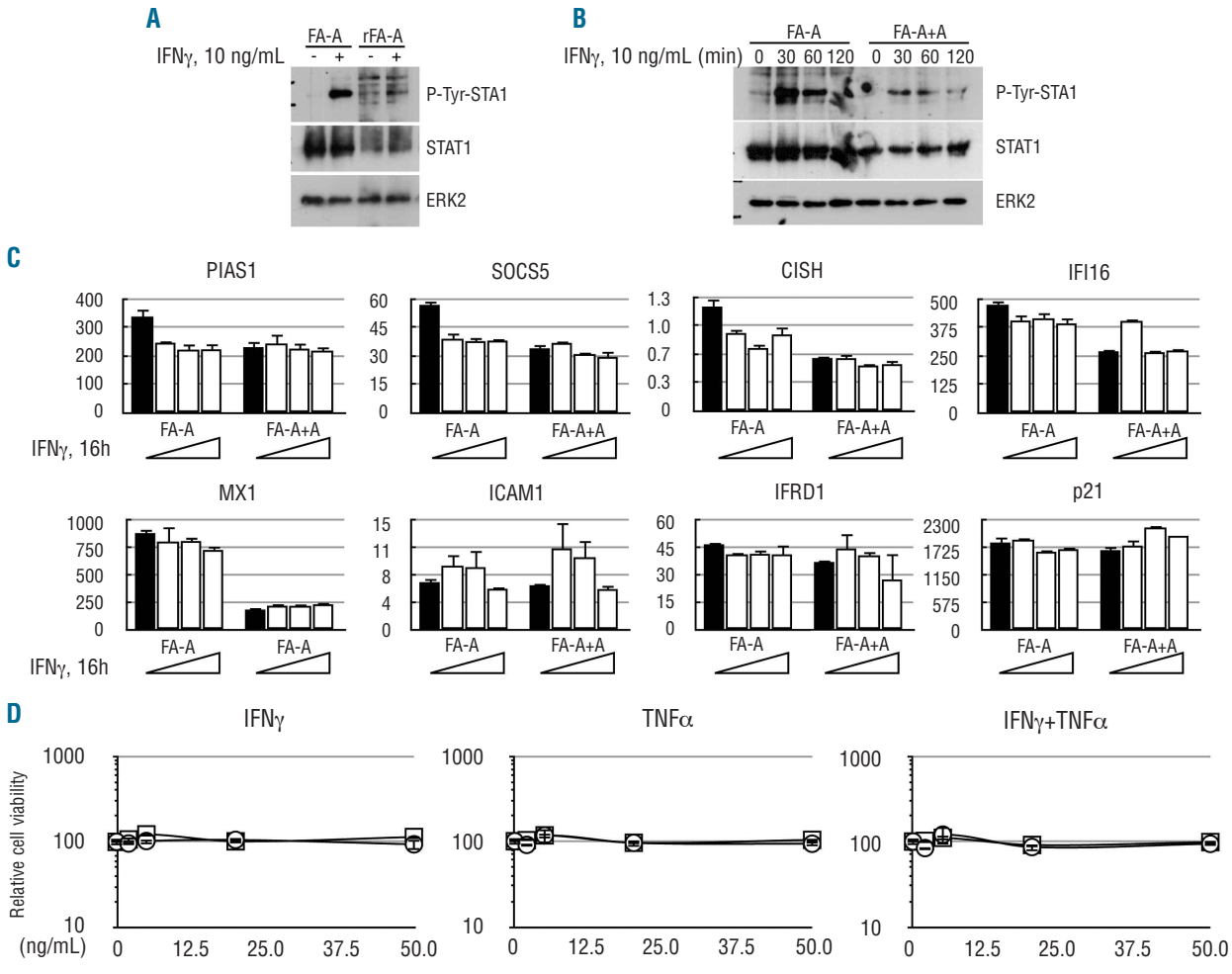

Figure 3. FA-A LCL reach higher levels of activated STAT1 but have an impaired response to IFNγ. (A) FA-A LCL or spontaneously reverted isogenic counterparts were treated with 10 ng/mL IFNγ for 30 min. Whole cell protein extracts were analyzed by western blot for activation of STAT1 using an anti-phospho-Tyr701-STAT1 antibody. STAT1 and ERK2 protein levels are shown to confirm equal loading. (B) To analyze the kinetics of STAT1 activation after IFNγ treatment in FA-A LCL or their FANCA-transduced counterparts, the cells were treated with 10 ng/mL for the indicated times. Whole cell protein extracts were then obtained and analyzed by western blot. Phosphorylation on tyrosine 701 of STAT1 was demonstrated using a specific antibody. STAT1 and ERK2 protein levels are also shown for comparison. (C) mRNA levels of the indicated STAT1-responsive genes were analyzed by quantitative RT-PCR of FA-A LCL or their FANCA-tranduced controls. Cells were treated with 5, 25 or 50 ng/mL IFNγ (white bars) for 16 h or left untreated (black bars). (D) XTT viability cell assays performed on FA-A (circles) or their isogenic FANCA-transduced LCL (squares) treated with increasing concentrations of IFNγ, TNFα or both for 3 days.

hematopoietic stem cells to cytotoxic cytokines. Hypersensitivity to inhibitory cytokines is a characteristic of FA hematopoietic stem cells, reported to be reproduced by FA-C LCL.[25] However, FA-A LCL did not show any change in viability in response to IFNγ, TNFα or a combination of both at doses as high as 50 ng/mL each (Figure 3D). This result is in agreement with the observation of Pang *et al.* who reported that, in contrast to FA-C fibroblasts, FA-A and FA-G fibroblasts do not exhibit any greater sensitivity than normal fibroblasts to IFNγ and double-stranded RNA treatment.[26] Surprisingly, STAT1 phosphorylation in tyrosine 701 proceeded normally in FA-A LCL (Figure 3A,B), in marked contrast to what has been reported for FA-C LCL.[10] Due to the overexpression of STAT1, IFNγ induces much higher levels of tyrosine-phosphorylated STAT1 in FA-A LCL. However, the pathway is defective as this stronger activation is not translated into greater DNA binding activity of STAT1 (*data not shown*) or higher expression of IFNγ-induced genes (Figure 3C), although some of them, such as *MX1* or *CISH1*, had higher basal levels in FA-A LCL. These results agree with previous observations made in FANCC-deficient cells.[23,27] In this respect, FA-A and FA-C LCL share this common failure in the induction of IFNγ responsive genes and also the fact that some of them show higher basal levels in FA cells. However, while FANCC-deficient cells fail in the activation of STAT1 by IFNγ, our data suggest that FANCA-deficient cells carry a failure in STAT1 transactivation functions. These results support the idea that FANC proteins have other functions apart from their participation in nuclear DNA repair protein complexes and provide a molecular basis for the phenotypic differences between FANCA and FANCC. The impairment in the response of this pathway, considered as an anti-viral pathway, could also be attributable to the interference of the Epstein-Barr virus used in the immortalization process to generate the

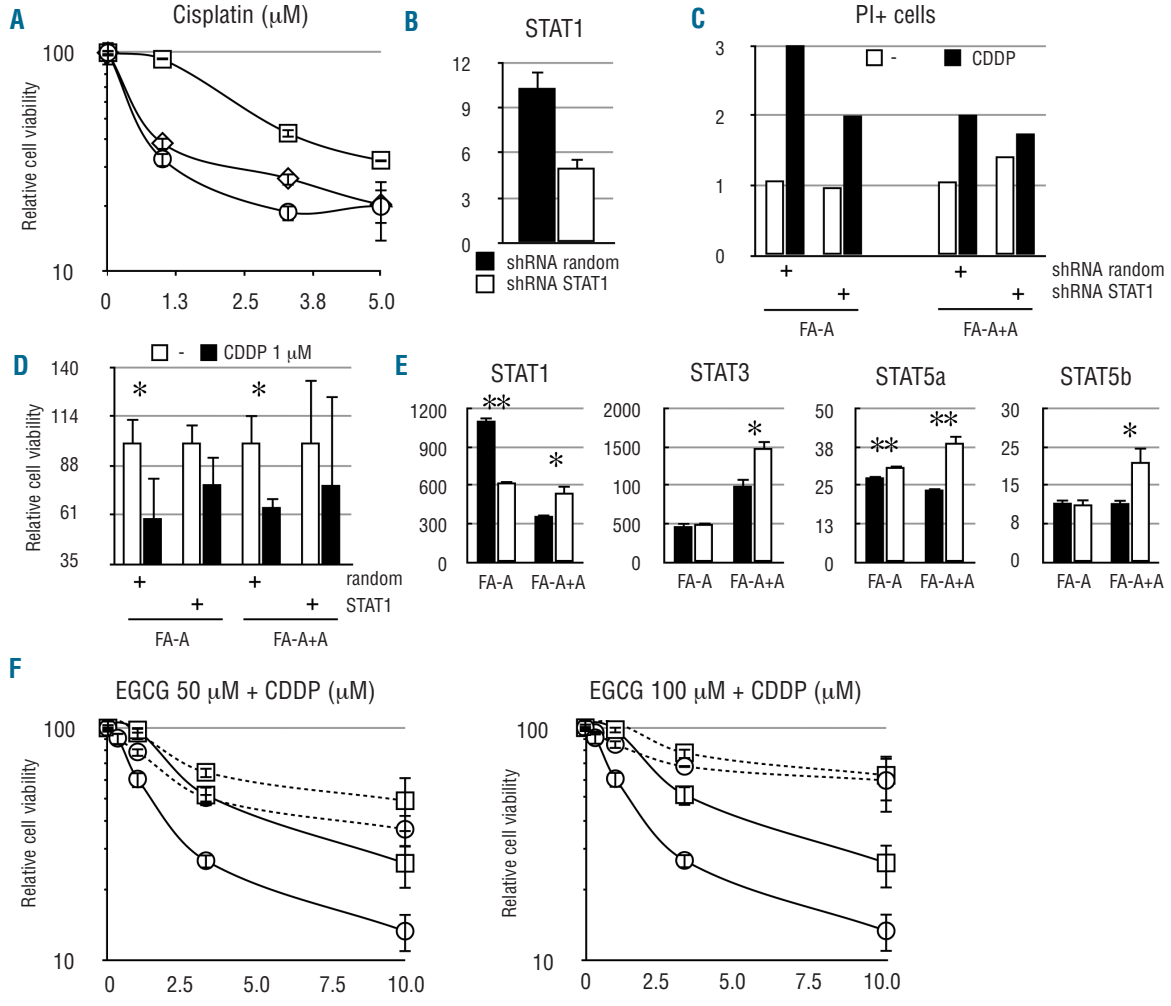

Figure 4. The elevated levels of STAT1 contribute to the sensitivity to DNA interstrand crosslinking agents of FA-A LCL. (A) Two different FA-A LCL were grown with increasing concentrations of cisplatin for 3 days. Their viability at each cisplatin concentration was then measured using a XTT viability cell assay. The viability curves of both FA-A LCL (diamonds and circles) and one of the cell lines transduced with FANCA cDNA (squares) are depicted. (B) Quantitative RT-PCR analysis of STAT1 mRNA expression in FA-A LCL transduced with either a shRNA random construct (black bar) or a shRNA against STAT1 (white bar). (C) shRNA random or shRNA STAT1-transduced LCL were treated with cisplatin (white bars) or left untreated (black bars). After 24 h, the cells were stained with propidium iodide and the number of red cells was quantified in a cytometer. A representative experiment of at least four different ones is depicted. (D) FA-A LCL or their isogenic corrected counterparts were transiently transfected with a non-target shRNA construct or a shRNA against STAT1 as indicated, sorted and then treated with 1 μM cisplatin (CDDP, black bars) for 3 days or left untreated (white bars). The graph depicts relative cell viability as measured by the XTT assay. The statistical significance of the data was tested by a paired t test and the reported *P* values are two-tailed (**P*<0.05, ***P*<0.01). (E) FA-A LCL and their FANCA-transduced counterparts were treated with epigallocatechin-3-gallate (EGCG) (white bars) or left untreated (black bars) for 24 h. Total RNA was extracted and the amount of the indicated mRNA was analyzed by quantitative RT-PCR. The statistical significance of the data was tested by a paired t test and the reported *P* values are two-tailed (**P*<0.05, ***P*<0.01). (F) XTT viability cell assay performed on FA-A LCL (circles) or their isogenic FANCA-transduced counterparts (squares), treated with increasing amounts of cisplatin for 3 days in the presence (dashed lines) or absence (solid lines) of the indicated concentrations of EGCG. Means ± standard deviations of triplicate values are given.

LCL. This idea is supported by the fact that no LCL showed variations in their viability in response to cytokines, independently of their FANC protein status.

Given that the most clear biological characteristic of the FA-A LCL was their greater sensitivity to DNA interstrand crosslinking agents (Figure 4A), we decided to investigate the role of STAT1 overexpression in this aberrant response. Many different observations have attributed STAT1 a function in the inhibition of cell growth and the promotion of apoptosis.[15,28] Our data demonstrate that the elevated levels of STAT1 in FA-A lymphoblasts correlate directly with these cells' sensitivity to cisplatin. These data are in agreement with those of previous studies showing that STAT1 interacts with and acts as a coactivator of p53 in the DNA damage induction of apoptosis.[13] Moreover, preliminary results from our laboratory indicate that interference of STAT1 expression using shRNA constructs impairs the accumulation of p53 after cisplatin treatment in FA-A LCL. This exacerbated p53 response to DNA damage has been demonstrated to reduce the hematopoietic stem cell pool in FA patients[7] and our data support a role of STAT1 in this process, possibly through the inhibition of MDM2.[13] Importantly, we describe a chemical inhibitor of STAT1, EGCG, which improves the viability of both

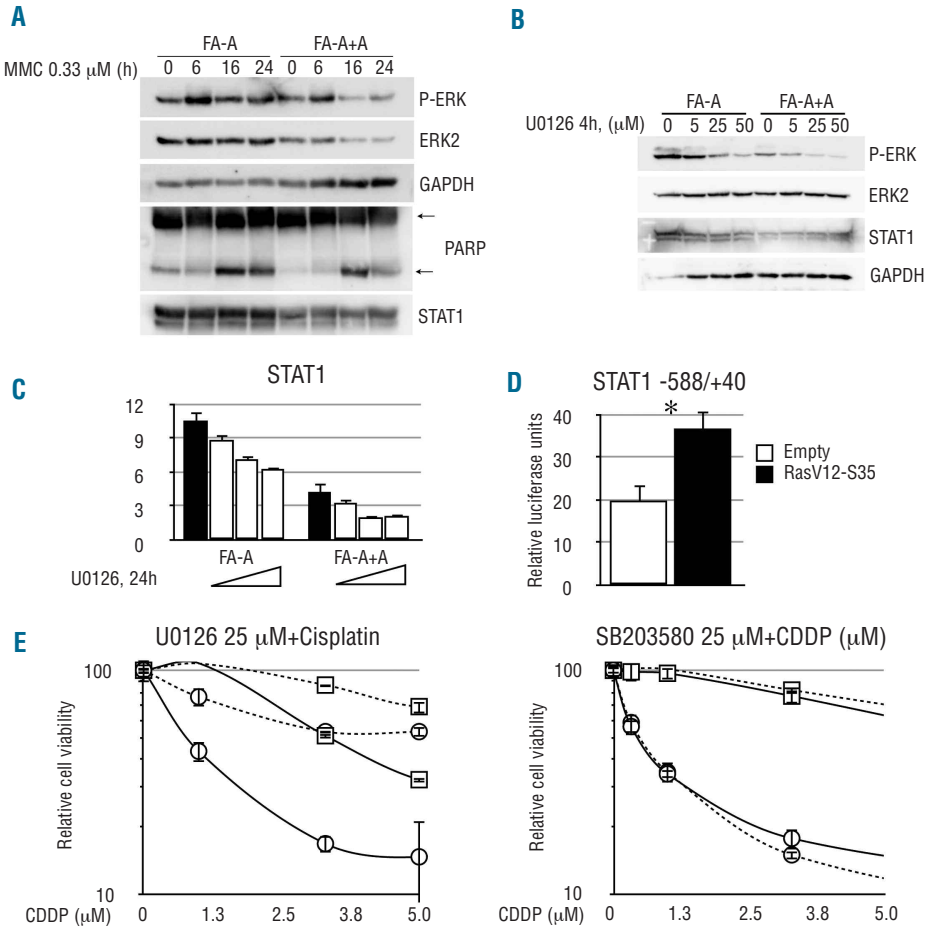

Figure 5. The constitutive activation of ERK in FA-A LCL is responsible for their overexpression of STAT1. (A) FA-A LCL or their corrected counterparts were treated with 0.33 μM mitomycin C (MMC) for the indicated times and their whole cell extracts were analyzed by western blot for the activation of ERK using a specific anti-phospho.ERK antibody. The blot was stripped and analyzed with other antibodies as indicated. (B) Western blot analysis of the activation of ERK and the accumulation of STAT1 in whole cell extracts from FA-A LCL and their FANCA-transduced counterparts treated with the indicated concentrations of U0126 for 4 h. (C) Quantitative RT-PCR analysis of the expression of STAT1 mRNA in FA-A LCL or their phenotypically corrected counterparts after a 24 h incubation in the presence of 0, 5, 25 and 50 μM U0126. (D) 293T cells were transfected with a reporter luciferase construct driven by the sequence between positions -588 and +40 relative to the transcription start site of the STAT1 gene, along with an expression vector for the V12-S35 mutant form of HRas. (E) XTT viability analysis of FA-A LCL (circles) or their corrected counterparts (squares) treated (dashed lines) or not (solid lines) with either U0126 or SB203580 and increasing concentrations of cisplatin (CDDP) as indicated. Means ± standard deviations of triplicate values are given.

FANCA-deficient and -proficient LCL in the presence of DNA interstrand crosslinking drugs (Figure 4). This opens a new therapeutic possibility for the treatment of progressive bone marrow failure of FA patients. Although EGCG has been reported to show specificity in the inhibition of STAT1,[20] the spectrum of described effects of this compound within the cell is quite broad and includes the prevention of oxidative damage, anti-angiogenesis, antitumor properties and modulation of cell response to chemotherapy.[29] All of these effects indicate that EGCG could be an excellent drug in the treatment of FA, although further studies are needed to establish its real effects on FA cells.

One of the molecular characteristics of FANCA-deficient cells is their constitutive activation of several MAPK pathways.[22] However, our previous data demonstrated a failure to activate JNK correctly in response to genotoxic stress in FA-A LCL.[30] Moreover, a screen of activated kinases in FA cells performed in our laboratory showed elevated levels of phosphorylated ERK and p38 but not significant variations in JNK activation in FANCA-deficient LCL (*unpublished results*). This led us to hypothesize a regulation of STAT1 by ERK in FA-A cells. It has been previously reported that the ERK pathway is involved in the accumulation and activation of STAT1 in myeloid leukemia cell lines.[24] We obtained evidence of such regulation by co-transfecting a luciferase reporter construct driven by a regulatory region of the STAT1 gene along with an expression vector for RasV12-S35, a mutant of Ras that specifically activates the Raf-ERK pathway.[31] Our data confirm the constitutive activation of ERK in FA-A LCL and how its inhibition leads to a reduction of both STAT1 mRNA and its coded protein. Moreover, treatment of FA-A LCL with U0126, an inhibitor of ERK activation, has a similar cisplatin-protecting effect as EGCG, further underlining the role of STAT1 overexpression in the sensitivity of these cells to cisplatin and opening up different ways to block its effect.

In summary, we report here that FANCA-deficient cells overexpress STAT1 due to the constitutive activation of ERK; this provides an explanation for the mixed sensitivity of FA cells to genotoxic stress and inhibitory cytokines. Most importantly, we report the action of two compounds that revert this sensitivity.

Acknowledgments

The authors would like to thank Drs. Isabel Badell (Hospital Sant Pau, Barcelona), Julián Sevilla (Hospital Universitario Niño Jesús, Madrid) and Jesús Estella (Hospital Infantil Sant Joan de Deu, Barcelona) for kindly providing us with bone marrow and peripheral blood samples. We also thank Rosario Gaitán for the management of the deliveries. FA lymphoblastoid cells lines and their corrected counterparts were kindly provided

by Drs. Juan Bueren from Ciemat, Madrid and Jordi Surrallés from UAB, Barcelona.

Funding

This work was supported by grants PS09/01533 from the Instituto de Salud Carlos III and UC08/03 from the Fundación Marqués de Valdecilla-IFIMAV and Universidad de Cantabria. IPR and DSC are recipients of a contract from the Instituto de Formación e Investigación Marqués de Valdecilla-IFIMAV.

Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

References

1. D'Andrea AD. Susceptibility pathways in Fanconi's anemia and breast cancer. N Engl J Med. 2010;362(20):1909-19.
2. Larder R, Karali D, Nelson N, Brown P. Fanconi anemia A is a nucleocytoplasmic shuttling molecule required for gonadotropin-releasing hormone (GnRH) transduction of the GnRH receptor. Endocrinology. 2006;147(12):5676-89.
3. Otsuki T, Nagakura S, Wang J, Bloom M, Grompe M, Liu J. Tumor necrosis factor-alpha and CD95 ligation suppress erythropoiesis in Fanconi anemia C gene knockout mice. J Cell Physiol. 1999;179(1):79-86.
4. Haneline LS, Broxmeyer HE, Cooper S, Hangoc G, Carreau M, Buchwald M, et al. Multiple inhibitory cytokines induce deregulated progenitor growth and apoptosis in hematopoietic cells from Fac-/- mice. Blood. 1998;91(11):4092-8.
5. Li J, Sejas DP, Zhang X, Qiu Y, Nattamai KJ, Rani R, et al. TNF-alpha induces leukemic clonal evolution ex vivo in Fanconi anemia group C murine stem cells. J Clin Invest. 2007;117(11):3283-95.
6. Ibáñez A, Río P, Casado JA, Bueren JA, Fernández-Luna JL, Pipaón C. Elevated levels of IL-1beta in Fanconi anaemia group A patients due to a constitutively active phosphoinositide 3-kinase-Akt pathway are capable of promoting tumour cell proliferation. Biochem J. 2009;422(1):161-70.
7. Ceccaldi R, Parmar K, Mouly E, Delord M, Kim JM, Regairaz M, et al. Bone marrow failure in Fanconi anemia is triggered by an exacerbated p53/p21 DNA damage response that impairs hematopoietic stem and progenitor cells. Cell Stem Cell. 2012; 11(1):36-49.
8. Anur P, Yates J, Garbati MR, Vanderwerf S, Keeble W, Rathbun K, et al. p38 MAPK inhibition suppresses the TLR-hypersensitive phenotype in FANCC- and FANCA-deficient mononuclear phagocytes. Blood. 2012;119(9):1992-2002.
9. Darnell JE, Kerr IM, Stark GR. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science. 1994;264(5164): 1415-21.
10. Pang Q, Fagerlie S, Christianson T, Keeble W, Faulkner G, Diaz J, et al. The Fanconi anemia protein FANCC binds to and facilitates the activation of STAT1 by gamma interferon and hematopoietic growth factors. Mol Cell Biol. 2000;20(13):4724-35.
11. Meraz MA, White JM, Sheehan KC, Bach EA, Rodig SJ, Dighe AS, et al. Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. Cell. 1996; 84(3):431-42.
12. Durbin JE, Hackenmiller R, Simon MC, Levy DE. Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. Cell. 1996;84(3): 443-50.
13. Townsend PA, Scarabelli TM, Davidson SM, Knight RA, Latchman DS, Stephanou A. STAT-1 interacts with p53 to enhance DNA damage-induced apoptosis. J Biol Chem. 2004;279(7):5811-20.
14. Townsend PA, Cragg MS, Davidson SM, McCormick J, Barry S, Lawrence KM, et al. STAT-1 facilitates the ATM activated checkpoint pathway following DNA damage. J Cell Sci. 2005;118(Pt 8):1629-39.
15. Kim HS, Lee M-S. STAT1 as a key modulator of cell death. Cell Signal. 2007;19(3):454-65.
16. Youlyouz-Marfak I, Gachard N, Le Clorennec C, Najjar I, Baran-Marszak F, Reminieras L, et al. Identification of a novel p53-dependent activation pathway of STAT1 by antitumour genotoxic agents. Cell Death Differ. 2008;15(2):376-85.
17. Pang Q, Christianson TA, Keeble W, Diaz J, Faulkner GR, Reifsteck C, et al. The Fanconi anemia complementation group C gene product: structural evidence of multifunctionality. Blood. 2001;98(5):1392-401.
18. Dufour C, Corcione A, Svahn J, Haupt R, Poggi V, Béka'ssy AN, et al. TNF-alpha and IFN-gamma are overexpressed in the bone marrow of Fanconi anemia patients and TNF-alpha suppresses erythropoiesis in vitro. Blood. 2003;102(6):2053-9.
19. Whitney MA, Royle G, Low MJ, Kelly MA, Axthelm MK, Reifsteck C, et al. Germ cell defects and hematopoietic hypersensitivity to gamma-interferon in mice with a targeted disruption of the Fanconi anemia C gene. Blood. 1996;88(1):49-58.
20. Menegazzi M, Tedeschi E, Dussin D, De Prati AC, Cavalieri E, Mariotto S, et al. Anti-interferon gamma action of epigallocatechin-3-gallate mediated by specific inhibition of STAT1 activation. FASEB J. 2001; 15(7):1309-11.
21. Townsend PA, Scarabelli TM, Pasini E, Gitti G, Menegazzi M, Suzuki H, et al. Epigallocatechin-3-gallate inhibits STAT-1 activation and protects cardiac myocytes from ischemia/reperfusion-induced apoptosis. FASEB J. 2004;18(13):1621-3.
22. Briot D, Macé-Aimé G, Subra F, Rosselli F. Aberrant activation of stress-response pathways leads to TNF-alpha oversecretion in Fanconi anemia. Blood. 2008;111(4):1913-23.
23. Fagerlie SR, Diaz J, Christianson TA, McCartan K, Keeble W, Faulkner GR, et al. Functional correction of FA-C cells with FANCC suppresses the expression of interferon gamma-inducible genes. Blood. 2001; 97(10):3017-24.
24. Zhou X, Fang Y, Jing H, Zhong L, Luo P, Song H, et al. Involvement of mitogen-activated protein kinase in signal transducer and activator of transcription-1 mediated differentiation induced by bortezomib in acute myeloid leukemia cells. Mol Carcinog. 2013;52(1):18-28.
25. Pang Q, Keeble W, Christianson TA, Faulkner GR, Bagby GC. FANCC interacts with Hsp70 to protect hematopoietic cells from IFN-gamma/TNF-alpha-mediated cytotoxicity. EMBO J. 2001;20(16):4478-89.
26. Pang Q, Keeble W, Diaz J, Christianson TA, Fagerlie S, Rathbun K, et al. Role of double-stranded RNA-dependent protein kinase in mediating hypersensitivity of Fanconi anemia complementation group C cells to interferon gamma, tumor necrosis factor-alpha, and double-stranded RNA. Blood. 2001;97 (6):1644-52.
27. Li Y, Youssoufian H. MxA overexpression reveals a common genetic link in four Fanconi anemia complementation groups. J Clin Invest. 1997;100(11):2873-80.
28. Dimco G, Knight RA, Latchman DS, Stephanou A. STAT1 interacts directly with cyclin D1/Cdk4 and mediates cell cycle arrest. Cell Cycle. 2010;9(23):4638-49.
29. Singh BN, Shankar S, Srivastava RK. Green tea catechin, epigallocatechin-3-gallate (EGCG): mechanisms, perspectives and clinical applications. Biochem Pharmacol. 2011;82(12):1807-21.
30. Pipaón C, Casado JA, Bueren JA, Fernández-Luna JL. Jun N-terminal kinase activity and early growth-response factor-1 gene expression are down-regulated in Fanconi anemia group A lymphoblasts. Blood. 2004;103(1): 128-32.
31. Krengel U, Schlichting I, Scherer A, Schumann R, Frech M, John J, et al. Three-dimensional structures of H-ras p21 mutants: molecular basis for their inability to function as signal switch molecules. Cell. 1990;62(3):539-48.