INVOLVEMENT OF STAT1 OVEREXPRESSION IN THE CELLULAR PHENOTYPE OF FANCONI ANEMIA GROUP A LYMPHOBLASTOID CELL LINES

BACKGROUND

Fanconi anemia (FA) is an autosomal recessive disease which was first described by the suisse pediatrician Guido Fanconi in 1927, who described the developmental abnormalities and the progressive bone marrow failure from two siblings. Since then, this disorder is known as Fanconi anemia and posterior observations settled that it is characterized by congenital malformations, progressive bone marrow aplasia, chromosomic aberrations and an extremely high predisposition to cancer, specially hematologic malignancies like acute myeloid leukemia (AML) and also solid tumors like head and neck carcinomas (1).

The high interest that many scientist have shown on this pathology led to the description of at least 14 different genes which mutation causes FA. Those genes determine the classification of patients in several complementation groups defined by letters (A, B, C, D1, D2, E, F, G, I, J, L, M, N, and P). Fanconi anemia group A is the major FA complementation group, accounting for 66% of the patients worldwide (2). The corresponding gene, FANCA, was cloned in 1996 (3, 4). This is a large gene consisting of 43 exons coding for a 160 kDa protein. Mutation analysis in FA-A patients did not identify mutational hotspots (5, 6). Surprisingly, there are not Fanconi anemia C patients in Spain, although it is a prevalent group in other countries.

The first genes described as related with Fanconi anemia codified previously unknown proteins, so they were named as FANC proteins. Others could be assimilated to previously known proteins involved in hereditary cancers (FANCD1 is BRCA2, FANCG is XRCC9, FANCI is KIAA1794, FANCJ is BRIP1, FANCL is PH9/POG, FANCM is Hef, FANCN is PALB2 and FANCP is SLX4).

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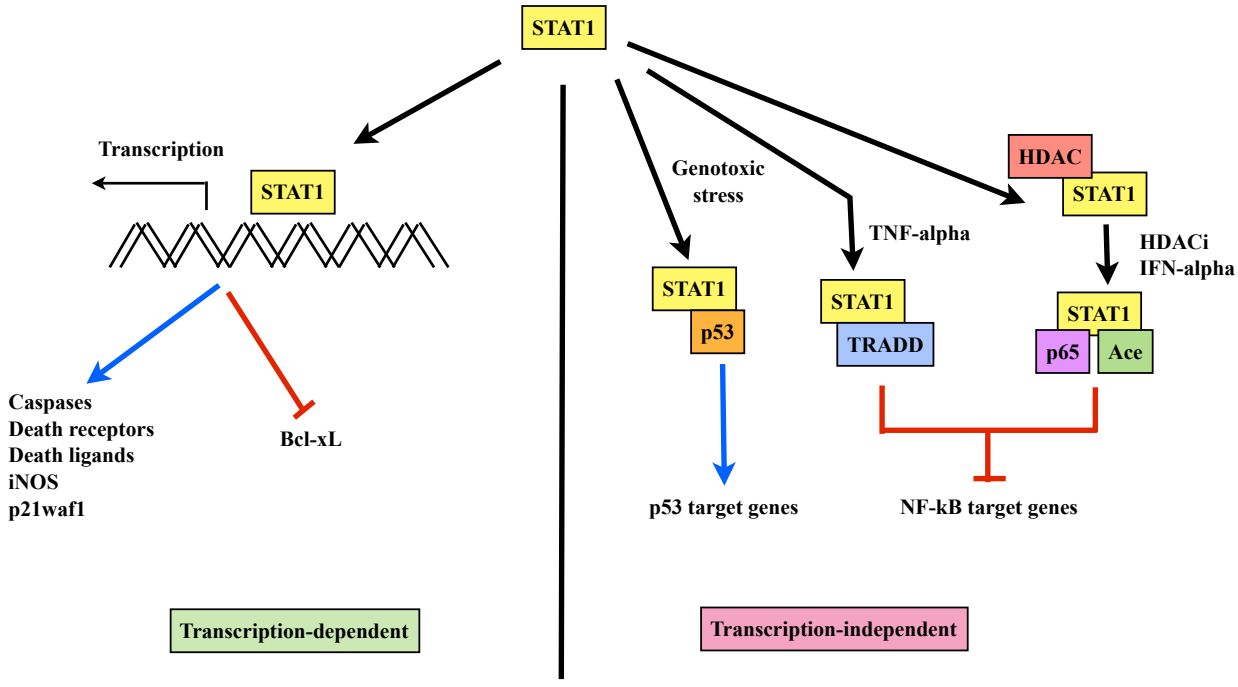
Protein-protein interactions studies determined that 9 of the FANC proteins formed a complex in the nucleus after DNA damage by genotoxic agents, such as mytomicin C and cisplatin. The high sensitivity to those compounds is an identity signal of the Fanconi anemia cells. The FA core complex then monoubiquitinate the FA-ID complex, formed by FANCD2 and FANCI proteins. Monoubiquitinated FANCD2 associates with BRCA protein and others involved in chromosome instability syndromes (Bloom Syndrome, Ataxia Telangiectasia, Nijmegen Syndrome, etc). Therefore, currently it is considered that all these proteins constitute a pathway named FA/ BRCA which plays an important role in the resolution of stalled replication forks that appear during normal DNA replication or by the action of DNA crosslinking agents.

Several reports have described the inhibition of Fanconi Anemia pathway to sensitize cells to cisplatin (7). Cisplatin is a typical chemoterapeutic agent used in several tumour treatments. This drug induces death of malignant cells by creating inter- and intrastrand DNA crosslinks (8) and may also induce DNA damage in normal cells (9, 10). Some investigations have demonstrated that cisplatin exposure induces inflammatory cytokine secretion by several cell lines (11, 12). Finally, different cell signaling proteins have been described as involved in cisplatin toxicity, such as STAT1 (13, 14), the tumor suppressor p53 and caspases, etc. STAT1 (signal transducer and activator of transcription 1) is a transcription factor involved in a diverse set of signaling cascades induced by cellular stress, growth factors and cytokines (15). STAT1 can be activated by phosphorylation at tyrosine 701 or serine 727 residue. Tyrosine phosphorylation is mainly induced by Janus kinase (JAKs) proteins and has specially been described after specific cytokines exposure such as Interferon gamma (15). IFN gamma has been described as involved

in innate immunity and as a potent antiproliferative agent on both normal and malignant cells. It acts via the sequential phosphorylation of JAKs and STAT1, as it is shown in the scheme below, inducing STAT1 homodimerization. Serine phosphorylation is required for maximal transcriptional activity of STAT1 and is catalyzed by different kinases such as ERK or p38 in response to different stress conditions such as DNA damage and exposure to ROS, TNF-alpha, etc. Several works have reported that STAT1 is an important cell death mediator after various types of cellular stress (16). STAT1 can regulate programmed cell death through both transcriptional-dependent and -independent mechanism, as it is shown in the scheme below (adapted from ref 15). Finally, it has also been reported that higher levels of STAT1 expression are related with an increase in the sensitivity to cisplatin of some cancer cell types (17).



Adapted from reference 15

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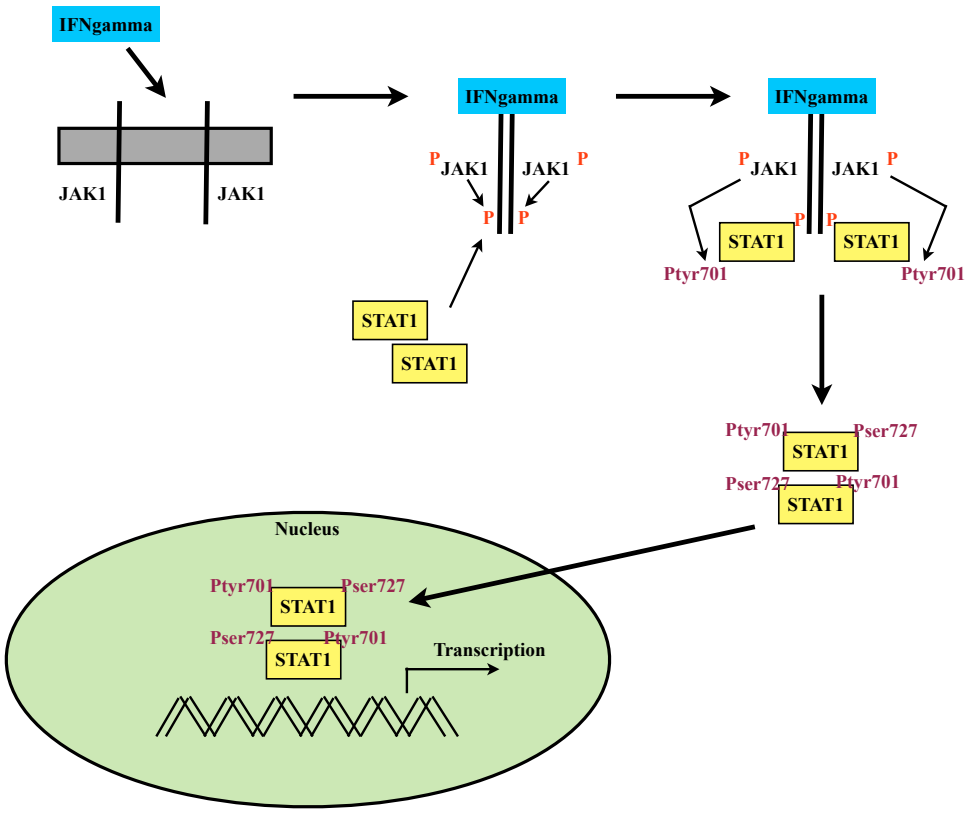
Fanconi anemia pathway has also been widely investigated in relation with the IFN gamma response via STAT1. In fact, FANCC protein has been related with the correct phosphorylation of STAT1 after exposure to this cytokine (**18**, **19**, **20**). Consequently, FA-C cells are unable to correctly activate the JAK-STAT pathway although paradoxically some of the target genes of this pathway are constitutively active in these cells (Mx1, p21, IRF-1). Functional correction of FA-C cells with FANCC suppresses the expression of interferon gamma-inducible genes. However, fibroblasts derived from Fanconi anemia group A patients have been reported to be unafected by TNFα (**21**), quite the opposite that occur with FA-C cells.



Adapted from reference 15

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OBJECTIVES

The main goal of our lab is the understanding of the cellular functions of the FANC proteins. This knowledge is crucial to find new therapies for the treatment of FA and cancers bearing spontaneous mutations of these genes. Previous data from our lab obtained from FANCA-deficient cells led us to the study of STAT1 activity in these cells.Stat1 activation has been shown to be defective in FA-C LCLs, so our first aim in this work was to study the response of FA-A LCLs to cytotoxic cytokines by analzying STAT1 protein levels, STAT1 phosphorylation status and its target genes.

On the other hand, as we found that STAT1 expression was markedly increased in FA-A LCLs, we stated another goal based on previous investigations that strongly related STAT1 overexpression with higher sensitivity to cisplatin. Based on this observation, we focused on the study of the role of STAT1 protein in the hypersensitivity of FA-A cells to crosslinking chemotherapeutic agents.

MATERIALS AND METHODS

Cell lines and Reagents. EBV-transformed human lymphoblasts from Fanconi Anemia group A were maintained in RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated FBS (Lonza), penicilin and streptomycin. Lymphoblastoid cell lines (LCLs) were grown in a humidified 5% CO2-containing atmosphere at 37ºC.

Bortezomib, a proteasomal inhibitor, was obtained from Velcade. Cisplatin was obtained from the Hematology Department. Human IFN gamma and Cycloheximide were obtained from Sigma. GammaBind G Sepharose was purchased from GE Healthcare.

Antibodies. ERK2 (c-14; Santa Cruz Biotechnology), STAT1 p48/p91 (e-23 X; Santa Cruz Biotechnology), pSTAT1 (Tyr 701-R; Santa Cruz Biotechnology), COUP-TFI (t-19 Xz; Santa Cruz Biotechnology), Ubiquitin (fl-76; Santa Cruz Biotechnology).

RNA isolation and processing. Total RNA was extracted from the cell cultures by use of Trizol (Invitrogen). Real-time quantitative PCR analysis involved the 7300 Detection System (Applied Byosistems). For RT-PCR, total RNA was extracted from cell lines by use of Trizol; cDNA was prepared with the Super Script kit from 1 µg of total RNA with oligo(dT) for analysis. Primers used are listed in Table 1.

Gene	Forward	Reverse	Assay
Stat1	AACAGAAAAATGCTGGCACC	AGAGGTCGTCTCGAGGTCAA	RT-PCR
beta2microglobuline	GAGACATGTAAGCAGCATCA	AGCAACCTGCTCAGATACAT	RT-PCR
Cish	GCCAGAAGGCACGTTCTTAG	GTACAAAGGGCTGCACCAGT	RT-PCR
Mx1	TGTGCAGCCAGTATGAGGAG	CCCACAGCCACTCTGGTTAT	RT-PCR
IRF1	TCCGGAGCTGGGCCATTCAC	GCGACAGTGCTGGAGTCAGGG	RT-PCR
IFRD1	TGCCCAATCAATGAAGTGAA	TCTGTTGGAAAATCCCGTTC	RT-PCR

Western Blotting. Cells were lysed in lysis buffer containgin 50 mM Tris-HCl, pH 8; 120mM NaCl; 0.5% Nonidet P-40; 2mM sodium orthovanadate; 100mM sodium fluoride; 1mM phenylmethylsulfonyl fluoride; 1% aprotinin; 1% leupeptin and 1mM DTT. Protein concentration was determined by the BCA Protein Assay Kit (Thermo Scientific). Total cell proteins were resolved on SDS/10% PAGE, transferred to PVDF membrane (Thermo Scientific), blocked in I-Block (Applied Biosystems), and incubated overnight with STAT1, (1:3000), pSTAT1 Tyr701 (1:500), and ERK2 (1:500) antibodies. Peroxidase-conjugated secondary antibodies were used at dilution of 1:10000. Signals were developed using the SuperSignal West Dura (Thermo Scientific).

Immunoprecipitation. Whole-cell lysates (1 mg of total protein) were incubated with the indicated antibodies (STAT1, COUPTFI) overnight at 4ºC. The immune complexes were bound to protein G Sepharose beads (2 hours at 4ºC) and washed 3 times with lysis buffer, followed by one wash with phosphate-buffered saline. Then samples were separated by SDS/PAGE and probed with Ubiquitin antibody (1:500).

Determination of protein stability. The turnover rates of proteins were determined by using cycloheximide to inhibit protein synthesis. Cells treated with 30μM cycloheximide (Sigma) were harvested at different time point after removing the cycloheximide inhibition. Equal amounts of cell lysates were subjected to SDS-PAGE and analyzed by immunoblotting to identify the Stat1 and Erk2 proteins.

Cell viability assay. The growth-inhibitory effect of both cisplatin and IFN gamma was examined by determining cell growth in parallel cultures in a 96-well plate. Cells seeded at 500 células/µL in a final volumen per well of 100 µL were continuously exposed to increasing concentrations of cisplatin or IFN gamma, as indicated. Cell numbers were evaluated after three days of culture. Growth inhibition is depicted relative to the number of cells in untreated controls culture wells that is set at 100%.

RESULTS

1. FA-A, but no FA-C, lymphoblastoid cell lines show overeprexpression of Stat1 at the protein level

Stat1 expression levels have only been studied in Fanconi anemia group C, but there are no reports analyzing the expression of Stat1 in Fanconi anemia group A (FA-A). To determine Stat1 expression levels in FA-A , whole cells protein extracts from lymphoblastoid cell lines (LCLs) derived from FA-A patients or healthy donors were analyzed by western blot in parallel (**Figure 1A**). Stat1 protein levels were markedly increased in FA-A LCLs compared to control LCLs. To elucidate if this difference in protein expression was due to a higher mRNA production, Stat1 transcriptional levels were analyzed in the same cell lines (**Figure 1B**). Interestingly, there were no differences in Stat1 mRNA levels between control and FA-A LCLs.

In order to assure that the data obtained were only due to the absence of a functional FANCA protein and there was no influence from the genetic background of each LCL, the same analysis was performed on 2 FA-A LCLs and their genetically corrected counterparts (**Figure 1C**, **1D**).

STAT1 protein and mRNA levels showed the same previous pattern when comparing FANCA-deficient cells to their corrected isogenic counterparts, demonstrating that the lack of a functional FANCA protein induces the STAT1 protein overproduction observed.

STAT1 overexpression seemed to be specific of FA group A, as we could assess by analyzing STAT1 levels in FA group C LCLs (**Figure 1E**), which did not show differences in STAT1 expression.

2. STAT1 shows longer half-life and reduced ubiquitination in FA-A LCLs than in their corrected LCLs

To investigate whether the differences observed in the STAT1 protein levels could be explain by a longer half-life of the protein, cycloheximide treatment was carried out in order to inhibit protein synthesis. FA-A LCL and its corrected counterpart were treated with 30μM cycloheximide and cells were collected at 3 different time points. Total protein extracts were analyzed by western blot using the anti-STAT1 antibody (**Figure 2A**). STAT1 protein kept high for 3 hours in FANCA-proficient cells with a sharp decrease between 3 and 17 hours after treatment. On the contrary, STAT1 levels kept almost unaffected after a 17 hours treatment with 30 μM cycloheximide in FA-A LCLs. This experiment demonstrates a stabilization of STAT1 protein in FA-A LCL.

As the Ubiquitin-proteasome system is a potential pathway by which STAT1 protein is degraded, we aimed to the inhibition of the proteasome to investigate the whether a defect in this process relies under the stabilization of STAT-1 in FANCA-deficient cells. Incubation of FA-A and

genetically corrected FA-A LCLs under 26nM of bortezomib for 1 hour followed by Coimmunoprecipitation studies were performed to asses the extent of ubiquitination-associated STAT1 under our treatment conditions (**Figure 2B**). In the setting of proteasome inhibition, there is a markedly reduction levels of STAT1-Ub in FA-A LCL than in cFA-A LCLs. This result suggests a possible defect in STAT-1 ubiquitination in FANCA-deficient cells.

3. IFNgamma treatment induces pTyr701 STAT1 but does not affect FA-A LCLs viability

As it has been described that FA-C LCLs show hipersensitivity to IFNgamma, and being Stat1 a central player in the pathway responding to this cytotoxic cytokine, we asked if the accumulation of STAT1 protein in FA-A LCL would be related to a stronger response of these cells to IFNγ. We used a viability assay to analyze the response of FA-A LCLs to a 3-days incubation in the presence of increasing concentrations of IFNγ. Interestingly, we observed no effect on cell viability even at the highest concentrations of the cytokine (50 ng/mL) used neither on control cells nor on FANC-deficient cells (**Figure 3A**).

Trying to elucidate if the absence of an effect of IFNgamma on LCL viability was due to a defect in STAT-1 activation, phosphorylation in the Tyr701 residue was analyzed in response to the cytokine (**Figure 3B**). FA-A and corrected FA-A LCLs were treated with 10ng/ml of IFNgamma and total protein was obtained at different time points after treatment in order to analyze pTyr701 STAT1 levels by western blot using a specific antibody. We observed that the phosphorylation of the tyrosine 701 occurred normaly in FANC-deficient LCLs. However, since STAT-1 protein levels were elevated in these cells, phospho-Tyr701-STAT-1 rised to a much higher levels in FA-

A LCLs that in their isogenic corrected counterparts. This result indicates that tyrosine 701 phosphorylation is not affected in FANC-deficient cells.

Since IFNγ treatment leads to a much higher levels of activated STAT-1, we asked if this will result in stronger activation of STAT-1 target genes in FA-A LCL. We analyzed the basal and induced expression of several STAT-1 target genes in FA-A LCLs after IFNγ treatment by RT-PCR. (**Figure 3C**). Although Mx1 and IRF1 have been reported to constitutively induced in FA cells of several complementation groups (referencias), we could not observe any variation in their basal mRNA levels in FA-A LCL. However, our experiments showed elevation in the CISH and IRFD1 mRNA basal levels in these cells. Treatment with increasing concentrations of IFNγ induced the accumulation of IFRD1 mRNA in FANCA-proficient cells, but FA-A LCLs showed no increase in any of the genes analyzed. These results indicate that even when Tyr701-phosphorylated STAT1 reaches much higher levels in FANC-deficient cells that in their isogenic corrected counterparts, there is a limited transduction of the signal to STAT1 target genes.

4. Cisplatin treatment induces STAT1 protein levels

Hypersensitivity to crosslinking agents as cisplatin is a hallmark of FA cells. Since STAT1 has recently been involved in different types of cell death in response to chemotherapeutic DNA-damaging agents

As it has yet been described in Fanconi anemia group A cells, these show hypersensitivity to cisplatin, as it is confirmed by an XTT assay (**Figure 4A**). In order to know if STAT1 may be involved in the exacerbated response to cisplatin in FA-A LCLs comparing with cFA-A LCLs,

both cell lines were treated with 3.3μM of cisplatin during 3 days (**Figure 4B**). Total protein extracts were analyzed by western blot, incubating the PVDF membrane with an anti-STAT1 antibody. Cisplatin induces STAT1 protein in FA-A LCL.

DISCUSSION

Unraveling of the molecular mechanism underlying cross-linking agent hypersensitivity and altered IFN gamma response in FA-A cells will help to provide more information about the function of the FA pathway and to place the FancA gene in the context of cell death and immune regulatory pathways. In this work, the relationship between the overexpression of Stat1 protein and the response to IFNgamma and the hypersensitivity to cisplatin was evaluated in FA-A lymphoblastoid cell lines. Searching for the molecular basis of FA-A cellular phenotype in a

possible alteration in the JAK-STAT1 signalling pathway, as it has been reported to happen in FANCC-deficient cells (**18**, **19**, **20**, **21**), we observed a strong elevation in STAT1 protein levels in LCL derived from FA-A patients. Most of the studies that have stablished JAK-STAT pathway as a key mediator of FA phenotype used cells from the FA complementation group C. Moreover, FANCC was stablished as a necessary protein for the correct activation of STAT1 in response to interferon and hematopoietic growth factors. Thus, STAT1 signalling pathway is somehow defective in FA-C cells, although paradoxically they show higher basal expression of several STAT1 target genes. Very little is known about the role of the JAK-STAT1 pathway in FA cells

from complementation group A and this is the first time that STAT1 is described as overproduced in FA-A lymphoblasts.

STAT1 overexpression was decreased when FA-A LCL were phenotypically reverted by transduction of a wild type FANCA cDNA. This gives more strength to the remark, pointing that the difference is only due to the absence of a functional FANCA protein.

Interestingly, STAT1 overexpression was only seen in FA group A cell lines and no in FA group C ones. This observation suggests a differential role of FANCA protein in comparison with FANCC one, at least in our cellular model. In this same direction is the observation that human FA-A LCLs are susceptible to TRAIL ligand when FA-C are not (22, 23). In addition, our data suggests and supports an increasing tendency that is giving evidence about more diverse biological functions of the Fanconi Anemia pathway proteins other than DNA repair.

As the STAT1 basal levels are higher, could be of interest to know if some STAT1 target genes are also induced at ground state, but surprisingly, we only found two up-regulated genes, CISH

and IFRD1. Although when IFN gamma treatment was carried out, CISH levels were not induced, suggesting that a non-STAT1 pathway is involved in constitutive activation of CISH in FA-A mutant cells. IFRD1 was up-regulated in FA-A LCLs at basal level, and was induced by interferon gamma only in control LCLs, not in FA-A mutant cells. This observation matches with the ones made in FA-C cells where some IFNγ genes are constitutively activated although treatment with the cytokine fails to induce their transcription (18, 19).

On the other hand, two common STAT1 target genes such as Mx1 and IRF1 showed no difference at mRNA levels between FA-A and control LCLs. In addition, after interferon gamma treatment neither Mx1 or IRF1 mRNA levels were induced. This observation contrasts with others reported by several groups, where Mx1 was elevated in FA cells from several complementation groups (24). IRF-1 has also been described as expressed at high levels in FANCC-deficient cells (25). The difference with our observations could, at least in this case, be attributable to differences in the function of individual FANC proteins within the cell, as other observation has suggested (22).

On the contrary to what is described in FA-C cells, where ubiquitination pathways were in general up-regulated (26), we found that overexpression of STAT1 protein, fact that could not be explained by a transcriptional mechanism since mRNA levels did not show any variations, was due to a reduced ubiquitination of STAT1. This had as a consequence a higher stability of the protein, as it was shown by a protein synthesis inhibitor assay, using cycloheximide. Thus, this suggested a possible role of the FANCA protein in the ubiquitination pathway.

Another feature found in FA-A LCLs was that they were not affected by IFN gamma treatment as it is shown in our cell viability assays. No significative differences were observed between the FA-A mutant and control cells. This result was yet described in FA-A human fibroblasts (21). But unlike of what was observed in FA-C cells, where the presence of a functional FANCC protein is necessary for the phosphorylation of STAT1 by JAK, phosphorylation status in Tyr 701 residue of STAT1 after IFN gamma treatment, was normal. This correlates with a functional FANCC protein in FA-A LCLs. However, this leads to a higher level of Tyr701-phospho-STAT1

that should result into stronger effects of IFNγ in FA-A LCLs. Why the lack of a functional FANCA protein impairs normal signaling by STAT1 is something under investigation in our laboratory. Taken all these observations into account, these data suggest that FANCA protein could somehow be involved either in nuclear import of STAT1 after its activation or in the correct STAT1 binding to its consensus sequence in DNA in order to induce transcription of its target genes.

Since high expression of STAT1 has been extensively related with a higher sensitivity to cisplatin, we wanted to know if it was involved in the hypersensitivity of FA-A cells to this drug. What we discover was a differentially up-regulation of the protein of STAT1 in FA-A LCLs, not observed in FANCA-proficient cells. This result suggests that the failure in DNA repair characteristic of FA-A cells might trigger DNA damage activated pathways that ultimately induce STAT1 protein accumulation. However, FA-C cells do not show STAT1 accumulation, so other molecular mechanisms might be involved. In this respect, we have observed a defective STAT1 ubiquitination in FA-A, so FANCA could be involved in STAT1 protein degradation as part of a regulatory mechanism for STAT1 signalling. The stabilization of STAT1 protein correlated with a differential reduction in FANCA-deficient cell viability.

In summary, we have observed a overexpression of STAT1 protein in FA-A LCLs, whichs seems to be due to an ubiquitination deffect. In order to relate this observation with the cellular phenotype of FA-A LCLs, we have studied, on one hand, the possible relation between cisplatin hypersensitivity shown by these cells and the high levels of STAT; and on the other hand, the response to interferon gamma of these lymphoblasts. We found of marked interest these two lines

of work because both chemical compounds have been extensively described to perform their roles through the activation of STAT1.

Taking our observations into acount, we state several essential future aims, focusing above all in the interference by shRNA of STAT1 in our cellular model, in order to elucidate if it plays a direct role in the hypersensitivity to cisplatin that FA-A LCLs show.

CONCLUSIONS

1.STAT1 is overexpressed at the protein level in FANCA-deficient LCLs but its messenger levels are not affected

2.STAT1 shows higher stability and reduced ubiquitination in FA-A LCLs

3.Despite IFN-gamma treatment induces higher levels of P-Tyr701 STAT1 in FA-A LCLs than in cFA-A LCLs, this does not correlate with a reduction in the number of viable cells

4.STAT1 protein is induced by cisplatin treatment and this induction seems to be stronger in FA-A LCLs, suggesting a possible role of STAT1 in their cisplatin hypersensitivity

5.The levels of STAT1 protein may serve as indicators of the sensitivity degree of FA-A LCLs to cisplatin

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FIGURE LEGENDS

1. STAT1 mRNA and protein basal levels in control, FA-A and corrected FA-A LCLs

A.STAT1 protein levels were analyzed by western blot in 4 control LCLs and 4 FA-A LCLs. ERK2 was used as loading control.

B.STAT1 mRNA levels were analyzed by RT-PCR in 4 control LCLs and 4 FA-A LCLs. Beta-2-microglobulin was used as housekeeping.

C.STAT1 protein levels were analyzed by western blot in 2 FA-A LCLs and their corrected counterparts. ERK2 was used as loading control.

D.STAT1 mRNA levels were analyzed by RT-PCR in 2 FA-A LCLs and their corrected counterparts. Beta-2-microglobulin was used as housekeeping.

E.STAT1 protein levels were analyzed by western blot in one FA-A LCL and its corrected counterpart, and one FA-C LCL and its corrected counterpart. Tubulin was used as loading control.

2. Analysis of STAT1 protein half-life and ubiquitination status in FA-A LCLs

A.Western blot of whole protein extracts from FA-A LCLs and its corrected counterpart treated with cycloheximide. ERK2 was used as loading control.

B.Immunoprecipitation was performed with an anti-ubiquitin antibody (lanes 1-4) or inespecific control (lane 5), and immunoblots of the precipitated material were performed by the use of anti-STAT1 antibody. The proteasome inhibitor bortezomib was used at a concentration of 26 nM during 1 hour.

3. Analysis of the response to interferon gamma in FA-A LCL

A.Cell viability of FA-A LCLs and their reverted counterparts in response to increasing doses of interferon gamma. The results are depicted as the viability percentage of non treated cells.

B.Analysis of the STAT1 tyrosine-701 phosphorylation after treatment with interferon gamma for the indicated times. ERK2 levels are shown as loading control.

C.Analysis of several STAT1 target genes after interferon gamma treatment. Beta-2-microglobulin was used as housekeeping.

4. Analysis of STAT1 protein levels in FA-A LCL after cisplatin treatment

A.Cells were assayed for viability using XTT assay after treating for 72 hours with increasing doses of cisplatin (CDDP). Results are expressed as percentage of viable cells at the final point of the experiment .

B.STAT1 protein levels analysis by western blot in FA-A and cFA-A LCLs whole protein extracts with and without cisplatin treatment during 3 days at the indicated dose. ERK2 was used as loading control.

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