OVEREXPRESSION OF STAT1 MAY BE INVOLVED IN THE HYPERSENSITIVITY TO CISPLATIN OF FANCONI ANEMIA GROUP A LYMPHOBLASTOID CELL LINES

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Introduction

Fanconi Anemia (FA) is a rare autosomal disorder which is manifested at the cellular level by genomic instability and hypersensitivity to DNA crosslinking agents such as cisplatin (1) mainly due to defects in DNA repair. Fanconi anemia patients suffer developmental abnormalities, early-onset bone marrow failure and extremely high cancer incidence.

FA is a genetically heterogeneous disease and to date, 14 genes have been identified as involved in it (FANCA, -B, -C, -D1, -D2, -E, -F, -G, -I, -J,-L, -M, -N and -P). Fanconi anemia group A is the most common one, representing the 66% of all cases.

The FA pathway is mainly involved in the repair of DNA damage, being the FA core complex recruited to the sites of damage in the nucleus, where it monoubiquitinates the FA-ID complex (2), which then co-localizes with other proteins involved in DNA repair. FA cells are unable to resolve stalled replication forks generated by DNA cross-linking agents.

Cisplatin is a DNA cross-linking chemotherapeutic drug that induces cell death (3) mediated in certain systems by reactive oxigen species (ROS) and inflammatory cytokines. The transcription factor STAT1, a key player in cell death (4), has been involved in the transduction of these signals in several cell types, and its targeted mutation has been related to apoptosis inhibition and transformation. Elevated expression of STAT1 in certain tumors has been related to their higher sensitivity to cisplatin (5). On the other hand, the chemical inhibition of the FA/BRCA pathway has been used to sensitize tumors to cisplatin (6) and it has also been reported that those cancer cells with an altered FA/BRCA pathway show a better response to that chemoterapeutic agent.

Here we report that STAT1 is overexpressed in FA-A LCLs and we provide preliminary evidence of a link between this elevated expression and the hypersensitivity to cisplatin in these cells.

Experimental procedures

- **Lymphoblastoid cell lines** (LCLs) were derived from peripheral blood B cells that were infected with **Epstein Barr Virus** for their immortalization. LCLs from Fanconi Anemia group A (FA-A) and its corrected counterpart (cFA-A), transduced with a wild type FANCA-expressing retrovirus, were maintained in RPMI 1640 medium suplemented with 10% FCS and antibiotics.
- STAT1 protein half life was measured using 30μM **cycloheximide**, an inhibitor of protein biosynthesis in eukaryotic organisms, in a time course experiment.
- The **ubiquitination status** of STAT1 was assayed by treating the LCLs with bortezomib, an inhibitor of the proteasome, followed by **immunoprecipitation** with an ubiquitin antibody and detecting STAT1 by immunoblot with a specific antibody.
- FA-A and cFA-A LCLs were treated with interferon (IFN)-gamma at the indicated dose, for different times, in order to analyze the phosphorylation of STAT1 protein at the Tyr701 residue.
- **XTT cell viability assay** was performed to analyze the effect of IFN-gamma on the cellular viability and to demonstrate the reverted phenotype of the cFA-A LCLs by their higher resistance to cisplatin.
- FA-A and cFA-A LCLs were treated with 1μM cisplatin for the indicated times, and the induction of STAT1 levels were determined by western blot.

Results

1. STAT1 protein is overexpressed in FA-A LCLs.

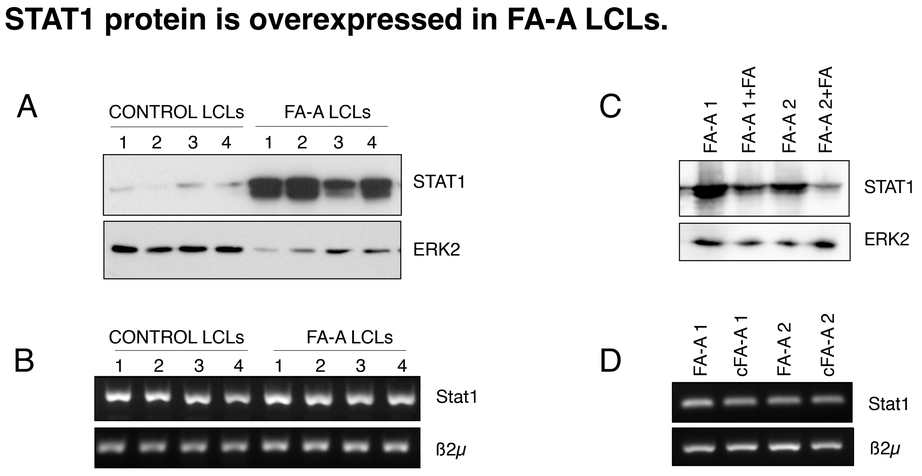

Figure 1. STAT1 mRNA and protein basal levels in control, FA-A and corrected FA-A LCLs.

A. STAT1 protein levels were analyzed by western blot in 4 control LCLs vs 4 FA-A LCLs. ERK2 was used as loading control.
B. Stat1 mRNA levels were analyzed by RT-PCR in 4 control LCLs vs 4 FA-A LCLs. Beta-2-microglobulin (ß2μ) 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 (ß2μ) was used as housekeeping.

2. STAT1 protein shows longer half-life and reduced ubiquitination in FA-A LCLs.

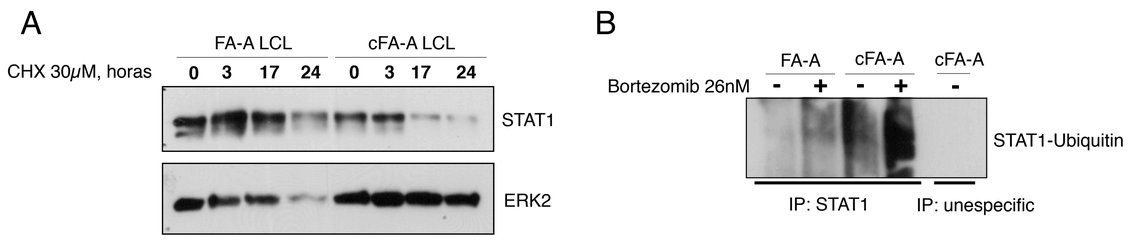

Figure 2. Analysis of STAT1 protein half-life and ubiquitination status in FA-A LCL.

A. Western blot of whole protein extracts from FA-A LCL and its corrected counterpart treated with cyclohexmide. ERK2 was used as loading control.
B. Immunoprecipitation was performed with an anti-ubiquitin antibody (lanes 1-4) or nonspecific 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 26nM during 1 hour.

3. FA-A LCLs do not show higher sensitivity to IFN gamma.

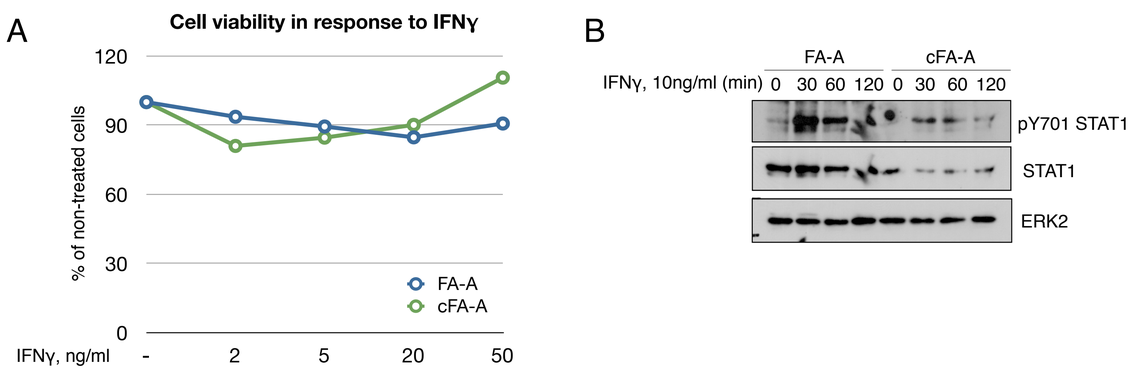

Figure 3. Analysis of the response to interferon gamma (IFNγ) in FA-A LCL.

A. Cell viability of FA-A LCLs and their reverted counterparts in response to increasing doses of IFNγ. The results are depicted as the viability percentage of non treated cells.
B. Analysis of the STAT1 tyrosine-701 phosphorylation after treatment with IFNγ for the indicated times. ERK2 levels are shown as loading control.

4. Cisplatin treatment induces STAT1 protein in FA-A LCLs

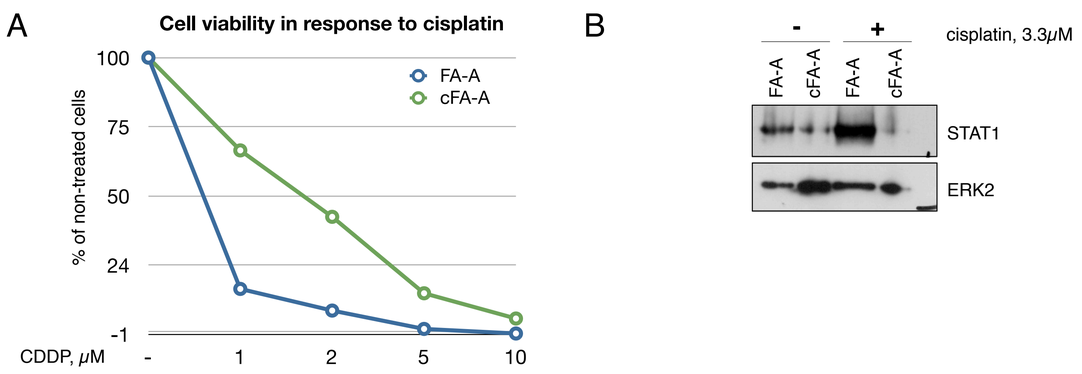

Figure 4. Analysis of STAT1 protein levels in FA-A LCL after cisplatin treatment.

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.

References

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Conclusions

- STAT1 is overexpressed at the protein level in FANCA-deficient LCLs but its messenger levels are not affected.
- STAT1 shows higher stability and reduced ubiquitination in FA-A LCLs.
- 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.
- 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.
- The levels of STAT1 protein may serve as indicators of the sensitivity degree of FA-A LCLs to cisplatin.

Future goals

- Knock-down by shRNA and the chemical inhibitor epigallocatechin gallate in order to study the role of STAT1 in the cisplatin hypersensitivity of FA-A LCLs.
- Elucidate the molecular mechanism that underlies the higher stability of Stat1 protein in FANCA-deficient cells.
- Increase the number of Fanconi Anemia group A patients in the study
- Study other FANCA-deficient cell types in order to determine if the levels of STAT1 follow the same pattern.

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