HLA-DR15 molecules derived from DRB1*1502 differ from those derived from DRB1*1501 in only one amino acid at position 86 (valine for DRB1*1502 and glycine for DRB1*1501) of the β-chain [31]. This structural similarity indicates that antigenic epitopes presented by these molecules are common [32,33]. For most autoimmune diseases where DRB1*1501 is associated with susceptibility in patients from Western countries, DRB1*1502 is expected to play the same role as DRB1*1501 in Japanese patients. However, in Japanese patients with multiple sclerosis, the frequency of DRB1*1502 is not increased in comparison to that in the controls [34,35]. As a result, DRB1*1502 appears to contribute to development of some autoimmune diseases via different mechanisms to DRB1*1501. In AA patients carrying DRB1*1501, certain antigens of which presentation requires position 86 of the β-chain to be glycine may likely induce an immune system attack to hematopoietic progenitor cells. It is also possible that DRB5*0101 and DRB5*0102, which are in complete linkage disequilibrium with DRB1*1501 and DRB1*1502, respectively, in the Japanese population [19] may be responsible for the difference because DRB5*0101 differs from DRB5*0102 by three amino acids in the antigen-peptide binding domain.

Our data may be relevant to the management of AA. Although the incidence of HLA-DR15 is significantly higher in AA patients than in the normal controls, only DRB1*1501 was found to be a predictive marker for a good response to ATG plus CsA therapy. AA patients with DRB1*1502 who do not show an increased proportion of PNH-type cells may not benefit from IST. HLA-DR typing has been considered to be useful for predicting a good response to IST in AA patients [7,8], but this costly test may not be necessary in the circumstance where the highly sensitive flow cytometry is available because the presence of a small population of PNH-type cells is the only significant factor that affects the response to ATG plus CsA therapy based on the findings of our multivariate analysis. Prospective studies are called for to confirm these findings.

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Minor population of CD55$^-$CD59$^-$ blood cells predicts response to immunosuppressive therapy and prognosis in patients with aplastic anemia

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We investigated the clinical significance of a minor population of paroxysmal nocturnal hemoglobinuria (PNH)–type blood cells in patients with acquired aplastic anemia (AA). We quantified CD55$^-$CD59$^-$ granulocytes and red blood cells (RBCs) in peripheral blood from 122 patients with recently diagnosed AA and correlated numbers of PNH-type cells and responses to immunosuppressive therapy (IST). Flow cytometry detected 0.005% to 23.1% of GPI-AP$^-$ cells in 68% of patients with AA. Sixty-eight of 83 (91%) patients with an increased proportion of PNH-type cells (PNH$^+$) responded to antithymocyte globulin (ATG) + cyclosporin (CsA) therapy, whereas 18 of 39 (48%) without such an increase (PNH$^-$) responded. Failure-free survival rates were significantly higher (64%) among patients with PNH$^+$ than patients with PNH$^-$ (12%) at 5 years, although overall survival rates were comparable between the groups. Numbers of PNH-type and normal-type cells increased in parallel among most patients with PNH$^+$ who responded to IST, suggesting that these cells are equally sensitive to immune attack. These results indicate that a minor population of PNH-type cells represents a reliable marker of a positive IST response and a favorable prognosis among patients with AA. Furthermore, immune attack against hematopoietic stem cells that allows PNH clonal expansion might occur only at the onset of AA. (Blood. 2006;107:1308-1314)

Introduction

Immunosuppressive therapy (IST) with antithymocyte globulin (ATG) plus cyclosporin (CsA) is the standard approach to treating acquired aplastic anemia (AA).[1-5] Approximately 70% of patients respond to this therapy and achieve remission. However, for the remaining 30%, IST might even be harmful because of an increased risk of opportunistic infections, particularly in the absence of any remission. The immune pathophysiology of patients should thus be understood at diagnosis, and IST should be applied only to those with immune-mediated AA. Several factors have been proposed as good markers that appear to reflect the immune pathophysiology of AA. These factors include an increased ratio of activated T cells,[6] increased interferon-γ expression in bone marrow,[7] and peripheral-blood T cells,[8] as well as increased expression of heat-shock protein 70.[9] Although these markers are useful in predicting responses to IST, few patients with AA have been tested, and the assays applied to detect these abnormalities are vulnerable to the effects of artifacts and the transportation of test samples. Consequently, none of the markers have been practically applied to predict responses to IST. Because of this, patients with AA are placed on IST without understanding the underlying pathophysiology.

One marker closely associated with immune pathophysiology in bone marrow failure is a small number of cells that are glycosylphosphatidylinositol-anchored membrane protein-deficient (GPI-AP$^-$), namely paroxysmal nocturnal hemoglobinuria (PNH)–type cells.[10-14] Dunn et al[11] have demonstrated that an increase in CD15$^-$CD66b$^-$CD16$^+$ granulocytes is associated with a good response to ATG among patients with myelodysplastic syndrome (MDS). Using 2-color flow cytometry that can distinguish proportions of CD55$^-$CD59$^-$CD11b$^+$ granulocytes and CD55$^-$CD59$^-$ glycophorin A$^+$ red blood cells (RBCs) below 0.1%, we also demonstrated that a population of 0.01% to 6% PNH-type cells among granulocytes and red blood cells predicts a response to CsA in patients with MDS.[15] Although one study group did not find a correlation between PNH-type cells and response to ATG in patients with AA,[14] an increase in the proportion of PNH-type cells was correlated with a good response to IST among our patients with AA[16] as well as those in another report.[12] However, the significance of a minor population of PNH-type cells in the management of patients with AA has remained obscure because the number of patients with recently diagnosed AA has been small and follow-up periods have not been long enough. Our sensitive flow cytometric protocol has not become popular despite its potential clinical usefulness, perhaps because of the lower cut-off values (0.003% for granulocytes and 0.005% for RBCs) than previous assays.[11,12,17,18]

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The outcome of IST in patients with AA is negatively affected by the length of time from diagnosis to treatment.[19] To clarify the role of a marker that would predict a good response to IST, the marker should be tested on patients who have been recently diagnosed with AA and before they receive therapy, and then the marker should be correlated with the subsequent response to IST. Since 1999, we have been studying the presence of PNH-type cells in peripheral blood using flow cytometry in 241 patients who had not yet undergone therapy and who were diagnosed with AA. The present study focuses on 122 patients who were treated with ATG and CsA within 1 year of the diagnosis of AA and compares the response rates to IST and subsequent survival between patients with (PNH^+) and without (PNH^-) an increased proportion of PNH-type cells. We also examined changes in the number of PNH-type cells after successful IST to characterize the immune system attack against hematopoietic stem cells that confers a survival advantage on PNH-type stem cells in immune-mediated AA.

Patients, materials, and methods

Patients

We evaluated PNH-type cells in peripheral-blood samples from 122 Japanese patients (55 men and 67 women; median age, 56 years) with idiopathic AA (75 severe and 47 moderate AA) before they received IST. The patients were diagnosed with AA at Kanazawa University Hospital, hospitals participating in a cooperative study led by the Intractable Disease Study Group of Japan, and other referring institutions. The severity of AA was classified according to the criteria proposed by Camitta et al.[20] All patients were treated with ATG Lymphoglobuline (Aventis Behring, King of Prussia, PA) 15 mg/kg/d, 5 days; plus CsA (Novartis, Basel, Switzerland) 6 mg/kg/d; within 1 year of diagnosis between April 1999 and December 2004. The dose of CsA was adjusted to maintain trough levels between 150 and 250 ng/mL, and the appropriate dose was administered for at least 6 months. Granulocyte colony-stimulating factor (G-CSF; filgrastim, 300 $\mu g/m^2$ or lenograstim, 5 $\mu g/kg$) was administered to some patients. Response to IST was evaluated according to the response criteria described by Camitta.[21] Complete response (CR) was defined as hemoglobin normal for age, neutrophil count more than 1.5×10^9/L, and platelet count more than 150×10^9/L. Partial response (PR) was defined as transfusion independent and no longer meeting criteria for severe disease in patients with severe AA, and it was defined as transfusion independence (if previously dependent) or doubling or normalization of at least one cell line or increase in baseline hemoglobin of more than 30 g/L (if initially less than 60 g/L), neutrophil count of more than 0.5×10^9/L (if initially less than 0.5×10^9/L), and platelet count of more than 10×10^9/L (if initially less than 20×10^9/L) in patients with moderate AA. The patients provided written, informed consent to participate in all procedures associated with the study, which was reviewed and approved by the ethical committee of Kanazawa University Hospital (study no. 46). The study also conforms to the recently revised tenets of the Helsinki protocol.

High-resolution 2-color flow cytometry

We improved the 2-color flow cytometry developed by Araten et al[22] as follows. Briefly, 3 to 5 mL heparinized blood was drawn from each patient. To detect PNH-type granulocytes, RBCs were lysed in NH_4Cl 8.26 g/L, $KHCO_3$ 1.0 g/L, and EDTA · E4Na 0.037 g/L (lysis buffer). After a saline wash, 50 μL leukocyte suspension was incubated with 4 μL phycoerythrin (PE)–labeled anti-CD11b monoclonal antibodies (mAbs; Becton Dickinson, Franklin Lakes, NJ), fluorescein-isothiocyanate (FITC)–labeled anti-CD55 mAbs (clone IA10, mouse IgG2a; Pharmingen, San Diego, CA), and FITC-labeled anti-CD59 mAbs (clone p282, mouse IgG2a; Pharmingen) on ice for 30 minutes.[13] To detect PNH-type RBCs, PE-labeled anti-glycophorin A mAbs (clone JC159; DAKO, Glostrup, Denmark) were included instead of anti-CD11b mAbs.[15] Fresh blood was diluted to 3% in phosphate-buffered saline (PBS), and then 50 μL was incubated with 4 μL PE-labeled anti–glycophorin A mAbs, FITC-labeled anti-CD55, and anti-CD59 mAbs on ice for 30 minutes. A total of at least 1×10^5 $CD11b^+$ granulocytes and glycophorin A^+ RBCs within each corresponding gate were analyzed using a FACScan (Becton Dickinson, Franklin Lakes, NJ) flow cytometry. To exclude damaged cells that often produce false-positive results, all samples were treated for flow cytometry within 24 hours after collection, and SSC^{dim} and $CD11b^{dim}$ granulocytes and glycophorin A^{dim} RBCs on the histograms were excluded from the analyses by careful gating as shown in Figure 1A. On the basis of analytic results from 68 healthy individuals, the presence of greater than 0.003% $CD11b^+$ granulocytes and 0.005% glycophorin A^+ RBCs was considered abnormal. Both thresholds greatly exceeded the mean + 4 SDs for GPI-AP^- granulocytes (0.0025%) and RBCs (0.0032%) determined in healthy individuals.[13,15] When PNH-type cells were increased in only 1 of the 2 cell lineages, another sample was collected, and the patient was deemed PNH^+ only when the second sample produced similar results.

We compared the sensitivity of detecting a few PNH-type cells in this manner with that of a low-resolution method[23] by analyzing the blood of some patients by 2-color flow cytometry using both PE-labeled anti-CD55

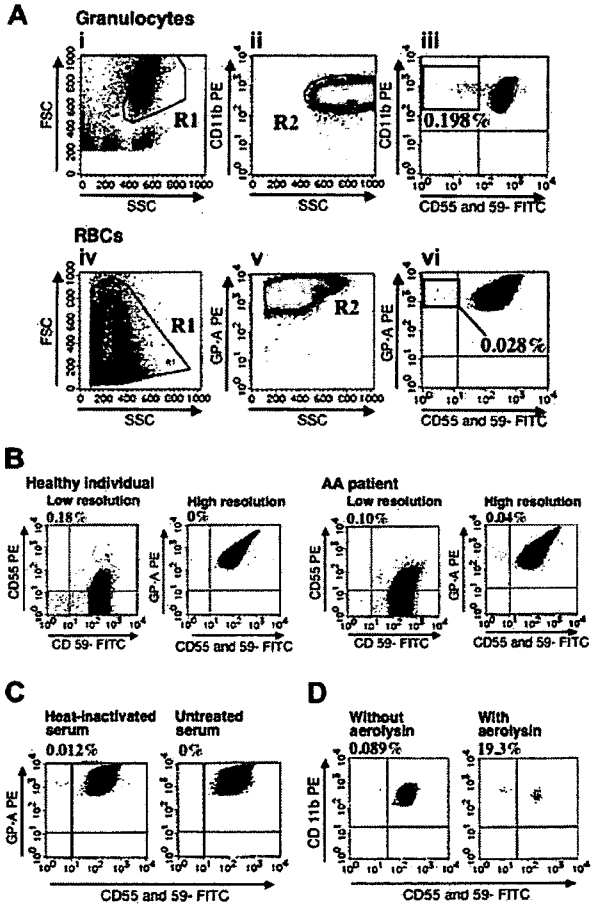

Figure 1. Validity of high-resolution flow cytometry. (A) An example of analysis on a patient with PNH^+ AA is shown. Gates were set up to exclude SSC^{dim} (i) and $CD11b^{dim}$ granulocytes and glycophorin A^{dim} RBCs (ii,v). Cells within rectangles showing horizontal distribution represent PNH-type cells. (B) RBCs from a healthy individual and a patient with AA were examined using a low-resolution assay and the high-resolution assay. Numbers on histograms denote the percentages of $CD55^-CD59^-$ cells in total RBCs for the low-resolution assay, and in glycophorin A^+ RBCs for the high-resolution assay. (C) RBCs from a patient with PNH^+ AA were incubated in acidified saline containing heat-inactivated or untreated serum.$CD55^-CD59^-$ RBCs were then quantified. (D) PNH^+ AA WBCs were incubated with or without 0.5×10^{-8} M aerolysin and analyzed by flow cytometry.

and FITC-labeled anti-CD59 mAbs. This assay defines the presence of 1% or more PNH-type cells as a significant increase.

Modified Ham test

Peripheral blood of patients with AA with a low proportion (< 0.1%) of $CD55^-CD59^-$ RBCs was washed with saline and suspended in saline at a hematocrit of 50%. The RBC suspension (15 μL) was incubated with 80 μL heat-inactivated fetal calf serum (FCS) for 10 minutes at 4°C for sensitization by anti–human heteroantibodies and then washed with saline. Human AB serum as a source of complement (0.5 mL) and 55 μL 0.2 N HCl were then added to the cell suspension. The negative control included heat-inactivated human AB serum instead of untreated human AB serum. These RBC suspensions were incubated for 60 minutes at 37°C and washed with PBS, and then the RBCs were analyzed by flow cytometry as described in "High resolution 2-color flow cytometry."

Aerolysin treatment of granulocytes

Peripheral blood from patients with AA with a low proportion of PNH-type granulocytes was lysed as described in "High resolution 2-color flow cytometry," and suspended in PBS at a density of 2×10^5 cells/mL. The leukocyte suspension was split into 2 portions; one was incubated for 15 minutes with and the other without 0.5×10^{-8} M aerolysin at 37°C.[24] Before and after the incubation with aerolysin, the suspension was examined by flow cytometry to detect $CD55^-CD59^-CD11b^+$ granulocytes as described in "High resolution 2-color flow cytometry."

Statistics

The Mann-Whitney test compared clinical characteristics between patients with PNH^+ and patients with PNH^-. Fisher exact test and logistic regression modelling[25] analyzed associations between individual pretreatment variables with response to IST. Kaplan-Meier methods graphically compared the cumulative incidence of the response with IST and time to event, and differences between patients with PNH^+ and patients with PNH^- were assessed by the log-rank test. A paired t test analyzed changes in the proportions of PNH-type cells associated with IST. All statistical analyses were performed using JMP version 5.0.1J software (SAS Institute, Cary, NC).

Results

Validity of high-resolution flow cytometry

Figure 1B shows that a low-resolution assay using PE-labeled anti-CD55 and FITC-labeled anti-CD59 mAbs detected greater than 0.1% PNH-type RBCs in the peripheral blood of a healthy individual, whereas our assay of the same sample detected 0% PNH-type cells. Thus, the low-resolution assay could not discriminate a patient with AA with 0.1% PNH-type cells from a healthy individual, whereas our method revealed 0.04% PNH-type RBCs in the same patient, indicating a diagnosis of PNH^+ AA. When the sensitivity of RBCs to complement-mediated lysis was examined using the modified Ham test, almost all RBCs in the glycophorin $A^+CD55^-CD59^-$ fraction disappeared after an incubation in acidified saline containing human AB serum, verifying the reliability of our method for detecting PNH-type RBCs (Figure 1C). Conversely, when granulocytes from a patient with PNH^+ AA were treated with aerolysin, approximately 99% of granulocytes in the $CD11b^+CD55^+CD59^+$ fraction disappeared, whereas almost all cells in the $CD11b^+CD55^-CD59^-$ fraction remained unchanged (Figure 1D), indicating that the few granulocytes in the $CD11b^+CD55^-CD59^-$ fraction had the properties of PNH-type cells.

Proportions of PNH-type cells in patients with AA

The proportion of PNH-type cells was increased in 83 (68%) patients. Among these patients with PNH^+, the number of PNH-type cells was increased in both the granulocytes and RBCs of 69 (83%) of them, in only the granulocytes of 12 (15%), and in only the RBCs of 2 (2%). Figure 2A shows the proportions of PNH-type granulocytes and histograms from 2 patients with PNH^+. Notably, the proportions of PNH-type granulocytes were below 0.1% in greater than 40% of patients with PNH^+. Table 1 compares the clinical characteristics between patients with PNH^+ and PNH^-. Although the PNH^+ group tended to be older and have higher WBC and MCV values than the PNH^- group, the clinical and hematologic parameters did not significantly differ between them.

Response to ATG and CsA therapy

Sixty-eight of 83 (91%) patients with PNH^+ improved with IST and achieved PR or CR at 12 months. However, only 18 of 39 (48%) patients with PNH^- responded to IST. Kaplan-Meier analysis showed that the chance of achieving PR was significantly better among patients with PNH^+ than among patients with PNH^- (Figure 3A). The rate of obtaining CR at 5 years was also significantly higher in patients with PNH^+ (36%) than in patients with PNH^- (3%) (Figure 3B). Multivariate analysis showed that among sex (male or female), age (older or younger than 40 years), severity (severe or moderate), presence or absence of chromosomal abnormalities, and presence or absence of increased PNH-type cells, only the presence of increased PNH-type granulocytes was a significant factor associated with good response to IST ($P < .001$). When patients with PNH^+ were classified into 5 subgroups according to the proportions of PNH-type granulocytes (0.003%-0.01% in 7, 0.01%-0.1% in 21, 0.1%-1.0% in 22, 1.0%-10.0% in 13, 10.0%-23.1% in 3), the response rates to IST at 6 months did not significantly differ (88%, 74%, 90%, 81%, and 100%, respectively) among these subgroups. The responses of all of these subpopulations were significantly better than that of patients with PNH^-.

Prognosis after IST

The median follow-up period was 26.4 months (range, 0.1 to 71.4 months). In contrast to the response rates, the rates of overall survival at 5 years were comparable between patients with PNH^+ (77%) and with PNH^- (71%) (Figure 4A). However, the probability of surviving failure free at 5 years was significantly higher in patients with PNH^+ (64%) than in patients with PNH^- (12%) when

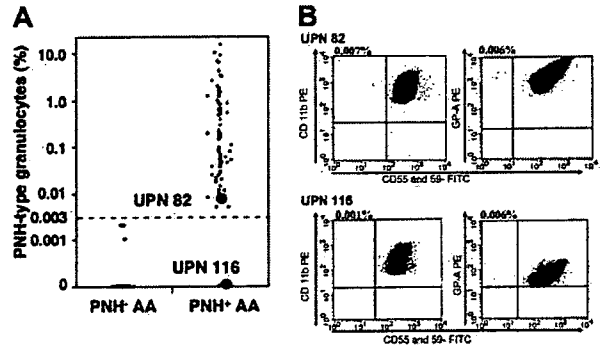

Figure 2. Proportions of PNH-type granulocytes. (A) Proportions of $CD55^-CD59^-$ granulocytes in each patient. (B) Histograms from one patient with PNH^+ (UPN 82) with minimal PNH-type cells and from another patient with increased PNH-type cells only in RBCs (UPN 116).

Table 1. Clinical characteristics of PNH⁺ and PNH⁻ patients

	PNH⁺	PNH⁻	P
No. of patients	83	39	NA
Median age, y (range)	57 (13-83)	54 (12-83)	.16
Sex, M/F	36/47	19/20	.58
Severity, severe/moderate	53/30	22/17	.43
Chromosome abnormality, no. of patients	7	3	.88
−7	0	1	
+8	2	1	
−Y	3	0	
Others	2	1	
Median WBC count, × 10^9 (range)	2.1 (0.5-4.3)	1.9 (0.7-3.2)	.15
Median neutrophil count, × 10^9/L (range)	0.53 (0.02-2.2)	0.49 (0.01-2.7)	.65
Median hemoglobin level, g/L (range)	67 (32-140)	67 (40-108)	.92
Mean corpuscular volume, fL (range)	101.5 (84.2-123.5)	98.5 (77.2-118.0)	.13
Median platelet count, × 10^9/L (range)	14.0 (2.0-60.0)	16.0 (1.0-87.0)	.65
Median reticulocyte count, × 10^9/L (range)	19.0 (3.0-90.0)	24.0 (2.0-106.0)	.50
Median time from diagnosis to IST, d (range)	30 (1-334)	33 (2-268)	.46
No. of patients who received G-CSF during IST	25	12	.94

NA indicates not applicable.

failure-free survival was calculated based on time to treatment failure. This was defined as whichever came first among time from the first day of treatment until salvage treatment for nonresponse, relapse, development of a clonal hematologic disease (PNH, MDS, leukemia), solid tumor, or disease- or treatment-related death (Figure 4B). Although the probability of evolution into florid PNH or MDS at 5 years after IST did not significantly differ between patients with PNH⁺ (6% and 3%) and patients with PNH⁻ (0% and 4%) (Figure 4C), the probability of relapse tended to be higher in patients with PNH⁻ (36%) than in patients with PNH⁺ (21%) (Figure 4D). Two (2%) patients with PNH⁺ and 7 (18%) with PNH⁻ underwent allogeneic bone marrow transplantation (BMT) from related (n = 6) or unrelated (n = 3) donors because of failure to respond to IST (n = 6) and relapse of AA (n = 3). Rates of survival after BMT did not significantly differ between the 2 groups (data not shown).

Changes in PNH-type granulocytes after IST

The presence of PNH-type cells after IST was serially tested in the peripheral blood of 53 of 122 patients. To characterize immune attack against hematopoietic stem cells that favors PNH-type cell clonal expansion, we examined the numbers of PNH-type cells in responsive patients. Figure 5A shows that the proportions of PNH-type granulocytes remained almost constant in 32 of 33 patients with PNH⁺ who responded to IST and decreased from 0.045% to 0% in only 1 patient (UPN 25). This indicates that the absolute number of PNH-type as well as of normal-type granulocytes increased in most responsive patients after IST. We compared the ratio of the degree of the increase in the absolute count between PNH-type (a) and normal-type (b) granulocytes before IST. The PNH-type granulocyte-to-normal-type granulocyte ratio in 32 patients ranged from 0.07 to 38.1 with a median of 1.06 (Figure 5B). The proportions of PNH-type cells did not change in 4 patients with PNH⁺ who were refractory to IST (Figure 5A-B). Sixteen patients with PNH⁻ were also tested after 6 to 24 months of IST. Only one patient who had achieved PR became PNH⁺ at 24 months and then relapsed with AA at 29 months after IST.

The proportions of PNH-type granulocytes were repeatedly determined in 23 patients for more than 24 months after IST. Figure 5C shows that the proportions remained constant over a long period in most patients including one (UPN 106) who had 0.1% PNH-type granulocytes (Figure 5D). The proportion of PNH-type granulocytes significantly increased from 3.31% to 76.0% in only one patient during the 4-year observation period.

Discussion

An increase in the proportion of PNH-type cells in peripheral blood has been implicated in the immune pathophysiology of bone marrow failure.[10] Several studies including our previous investigation found a correlation between an increase in the proportion of PNH-type cells and a favorable response to IST among patients with MDS[11,12,15] and with AA.[16,26] However, the clinical application of these findings has been hampered. Small patient cohorts and the relatively low prevalence of an increased number of PNH-type cells in these studies have led to concerns about unreliability of the correlation. The present study based on a larger number of patients with recently diagnosed AA conclusively demonstrated that a minor population of PNH-type cells predicts a good response to IST as well as good prognosis for patients with AA after IST.

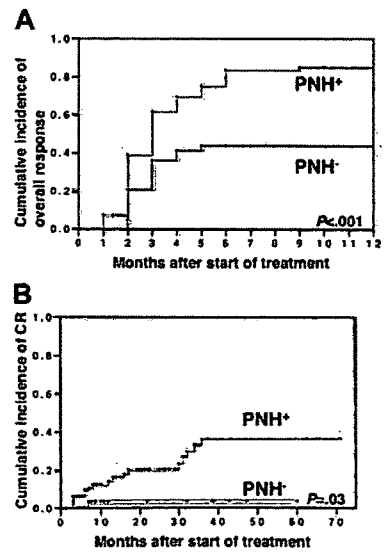

Figure 3. Response to immunosuppressive therapy. Incidence of overall (A) and complete (B) responses in patients with PNH⁺ and PNH⁻.

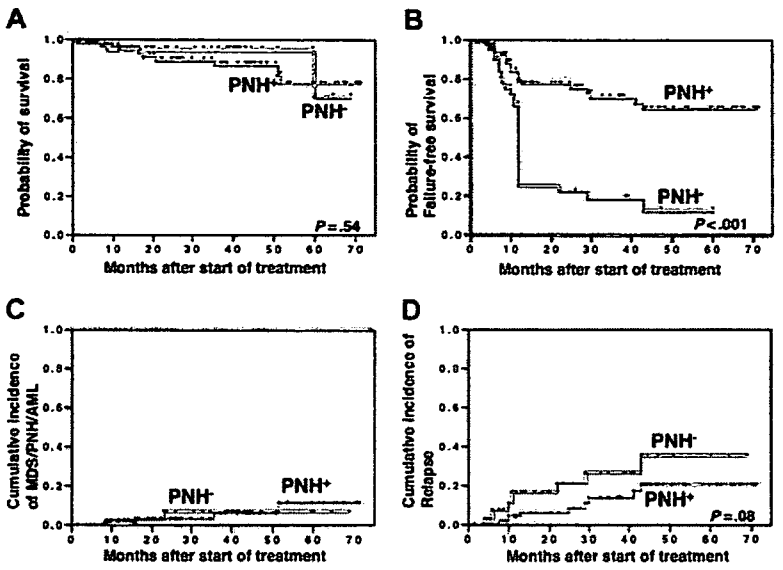

Figure 4. Prognosis after IST compared between patients with PNH$^+$ and with PNH$^-$. (A) Overall survival; (B) failure-free survival; (C) incidence of clonal hematologic disorders, including PNH, myelodysplastic syndrome, and acute myelogenous leukemia; and (D) incidence of relapse.

The reliability of our high-resolution flow cytometry, which was verified by the modified Ham test and by aerolysin treatment, revealed an increase in the number of PNH-type cells in 68% of the patients with AA. This was considerably higher than the reported prevalence.

The clinical features and overall survival rates did not significantly differ between patients with PNH$^+$ and patients with PNH$^-$ in the present study. However, failure-free survival was obviously better among patients with PNH$^+$ than patients with PNH$^-$. This indicated that, although patients with PNH$^-$ can survive as long as patients with PNH$^+$ after IST, they often require salvage or supportive treatment such as allogeneic stem cell transplantation and blood transfusions, because of a partial response to IST or a high rate of relapse. Contrary to the expectation based on the presence of abnormal hematopoietic clones such as PNH-type cells, the probability of evolving into clinical PNH or MDS in patients with PNH$^+$ was comparable to that in patients with PNH$^-$. The proportions of PNH-type granulocytes remained stable over a period of 1 to 66 months in most patients with PNH$^+$, a finding consistent with previous reports.[26,27] These findings indicate that

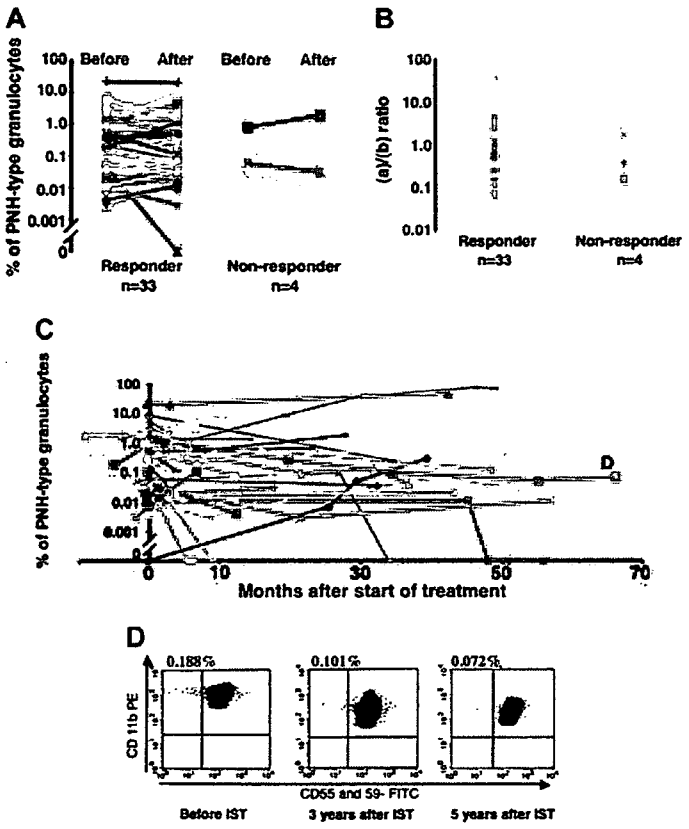

Figure 5. Changes in proportions of PNH-type granulocytes associated with responses to IST. (A) Change in responders and nonresponders. (B) Proportions of granulocyte counts after and before IST determined for PNH-type (a) and normal-type (b) granulocytes and ratios of PNH-type granulocytes (a) to normal-type granulocytes (b) were plotted. (C) Longitudinal analysis of PNH-type granulocytes. Proportions of PNH-type granulocytes of 37 patients with PNH$^+$ and 1 patient with PNH$^-$ who became PNH$^+$ (black line) were displayed. (D) Changes in proportions of PNH-type granulocytes over 5 years in patient UPN 106 with AA (shown as D in Figure 5C).

the presence of an increased proportion of PNH-type cells predicts not only a positive response but also a good quality of response to IST among patients with AA.

The significantly high response rate to IST among patients with PNH$^+$ AA suggests that PNH$^+$ AA is an authentic type of immune-mediated marrow failure. In line with this hypothesis, patients with PNH$^+$ AA often have a specific HLA-DR allele (HLA-DR15) and antigen-driven T-cell proliferation in the bone marrow.[12,28] Furthermore, antibodies against diazepam-binding inhibitor-related sequence-1 (DRS-1), a peroxisomal protein abundantly expressed by hematopoietic progenitor cells, are frequently detected in sera from patients with PNH$^+$ AA.[29] However, the relatively low response rate to IST among patients with PNH$^-$ AA indicates that a heterogeneous pathophysiology might underlie this subset of AA. In line with this notion as described in our previous study,[16] clonal hematopoiesis arose more frequently in patients with PNH$^-$ AA than in patients with PNH$^+$ AA. Even among patients who responded to IST, patients with PNH$^-$ AA rarely achieved complete recovery of hematopoiesis and were susceptible to AA relapse. Immune mechanisms that are not associated with an increase in the proportion of PNH-type cells might damage hematopoietic stem cells more profoundly than those in PNH$^+$ AA.

PNH-type stem cells might acquire a survival advantage over normal-type stem cells when T or natural killer (NK) cells attack hematopoietic stem cells.[30-32] The high response rate to IST in patients with PNH$^+$ AA indicates that such an immune mechanism is functional in this subset of AA. If the immune mechanisms were responsible for bone marrow failure, IST would more efficiently induce expansion of normal-type than of PNH-type stem cells. However, in most patients with PNH$^+$, successful IST resulted in a similar increase in the number of both PNH-type and normal-type granulocytes, which contradicts the immune escape theory. A similar finding has been reported by Maciejewski et al[26] for patients with AA with 1% or more CD15$^+$CD66b$^-$CD16$^-$ granulocytes. One possible explanation for this discrepancy is as follows. An immune attack against hematopoietic stem cells at the onset of AA that allows PNH-type stem cells to survive does not contribute to the subsequent progression of bone marrow failure, which is caused by different immune mechanisms targeting epitopes other than those that induce disease. Such epitope spreading occurs in the development of other immune diseases such as multiple sclerosis.[33] Alternatively, the suppression of hematopoiesis after the clonal expansion of PNH-type cells might be caused by myelosuppressive cytokines rather than antigen-specific T cells.

The presence of a few PNH-type cells has profound significance for the management of patients with recently diagnosed AA. Although those who have PNH$^-$ AA can improve with IST, the maximal response rate is 50% and the rate of failure-free survival at 5 years is below 20%. Therefore, allogeneic BMT is recommended more often than IST for young patients with PNH$^-$ who have HLA-compatible sibling donors. Conversely, IST is more frequently recommended than BMT for patients with PNH$^+$, particularly when the likelihood of BMT-related mortality is high. Among patients with AA who are unresponsive to the initial ATG and CsA therapy, those who benefit from a second IST might be PNH$^+$. Conventional flow cytometry capable of detecting 1% or more PNH-type cells would also be clinically useful in predicting response to IST because the response to IST does not change according to the proportion of PNH-type cells. The predictive value of an increased proportion of PNH-type cells for a favorable prognosis in AA identified here warrants a further worldwide prospective study on non-Japanese patients with AA.

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bjh short report

Preceding immunosuppressive therapy with antithymocyte globulin and ciclosporin increases the incidence of graft rejection in children with aplastic anaemia who underwent allogeneic bone marrow transplantation from HLA-identical siblings

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Summary

The incidence of graft rejection was determined in 66 children with acquired aplastic anaemia (AA) following bone marrow transplantation (BMT) from a related donor. Eleven of 65 evaluable patients experienced either early or late rejection. Multivariate analysis identified previous immunosuppressive therapy with antithymocyte-globulin (ATG) and ciclosporin (CsA) as a risk factor for graft rejection (relative risk: 16·6, $P = 0·001$). Patients who received ATG and CsA had a significantly lower probability of failure-free survival than those who did not (69·7 ± 6·2% vs. 87·9 ± 8·0%, $P = 0·044$). These results suggest that BMT should be instituted immediately in children with severe AA who have human leucocyte antigen-identical siblings.

Key words: bone marrow transplantation, immunosuppressive therapy, aplastic anaemia, childhood.

Immunosuppressive therapy (IST) is less expensive and has a lower associated incidence of treatment-related mortality and morbidity. Thus, several researchers have proposed evaluating IST as an initial therapy even in patients with severe aplastic anaemia (AA) who have sibling donors. This therapy would be followed by bone marrow transplantation (BMT) only in patients with inadequate response to IST or relapse (Lawlor *et al*, 1997; Fuhrer *et al*, 2005).

Since 1992, we have conducted a prospective multi-centre study of childhood AA (Kojima *et al*, 2000). In our study, BMT was used for patients with severe or very severe AA who had a human leucocyte antigen (HLA)-identical sibling. Combined IST with antithymocyte-globulin (ATG) and ciclosporin (CsA) was indicated for patients with non-severe AA, even if they had an HLA-identical sibling. However, 33 of 340 patients with severe or very severe AA received IST followed by BMT based on their family's choice, even if they had an HLA-identical sibling. This led us to examine the effect of preceding IST in AA patients who underwent BMT from an HLA-identical sibling to determine whether preceding IST has a detrimental effect on such patients.

Patients and methods

Sixty-six patients with acquired AA who underwent allogeneic BMT from an HLA-identical sibling between November 1992 and September 2004 were enrolled in the study. The diagnosis and assessment of disease severity were established according to published criteria (Camitta *et al*, 1979). The patients were aged between 0 and 18 years (median: 9 years), and 54 had received some form of specific treatment for AA before transplantation, including the following: ATG, CsA, and granulocyte colony-stimulating factor (G-CSF; $n = 21$); ATG and CsA ($n = 12$); G-CSF alone ($n = 10$); CsA with or without steroids ($n = 6$); and CsA with or without steroids and G-CSF ($n = 5$). The following conditioning regimens were used: cyclophosphamide (200 mg/kg) and ATG (Thymoglobulin, Sangstat, Lyon, France: 10 mg/kg) in 39 patients; fludarabine (120 mg/kg), cyclophosphamide (3000 mg/m^2) and 3 Gy of total-lymphoid-irradiation (TLI) or thoraco-abdominal-irradiation in 14 patients; cyclophosphamide (200 mg/kg) and TLI or total-body-irradiation in eight patients; and other regimens in five patients. Graft-*versus*-host-disease (GVHD) prophylaxis, consisting of a combination of methotrexate (MTX) and CsA, was used in 54 patients. The remaining patients received GVHD prophylaxis consisting of CsA, CsA plus steroids, or tacrolimus plus MTX.

Definitions

Failure-free survival (FFS) was defined as survival with sustained engraftment; death and graft-rejection were categorised as treatment failures. Studies on donor chimaerism were performed at each participating hospital by fluorescence *in situ* hybridisation of Y chromosomes or by study of the genetic polymorphism of variable numbers of tandem repeat short DNA sequences of marrow or peripheral blood cells. We assessed complete or mixed donor chimaerism according to previously described criteria (Hoelle *et al*, 2004). Treatment response following BMT is same as response after IST and has been described previously (Kojima *et al*, 2000).

Statistical analysis

Analyses of overall survival and FFS were performed using the Kaplan–Meier method, with differences compared by log-rank and Wilcoxon test. In univariate analysis, the χ^2-test and Fisher's exact test were used to assess risk factors for graft-rejection. P-values $< 0{\cdot}05$ were considered significant. Multivariate stepwise regression was performed to explore the independent effects of variables that showed a significant influence in univariate analysis.

Results

One patient died of septicaemia before the evaluation of engraftment. Of the 65 evaluable patients, two (3%) failed to engraft, and late graft-rejection occurred in nine (14%) of the 63 engrafted patients. The median time to late graft-rejection was 130 days after BMT (40–413 days). Thus, 11patients (17%) experienced early or late graft-rejection: among these patients, one showed recovery of autologous marrow function; two of four patients achieved partial recovery of bone marrow function following donor lymphocyte infusion; and five underwent successful second bone marrow grafts. Of the nine patients in whom donor chimaerism was studied, eight were mixed chimeras at the time of graft-rejection (15–90% donor type), and the remaining patient was a complete chimera with 100% donor-type haematopoietic cells.

In the univariate analysis, two variables were associated with graft-rejection: (1) history of IST with ATG–CsA and (2) the method of GVHD prophylaxis. Patients who received preceding therapy with ATG–CsA had a significantly higher incidence of graft-rejection compared with those who did not receive this therapy ($P = 0{\cdot}044$). Similarly, patients who received GVHD prophylaxis other than CsA and MTX had a significantly higher incidence of graft-rejection ($P = 0{\cdot}022$). In the multivariate analysis, the same variables remained statistically significant; the relative risk of graft-rejection was much higher in patients who received ATG–CsA than in those who did not (relative risk, 16·6; 95% confidence interval, 1·87–146·7; $P = 0{\cdot}001$).

There were considerably more cases of rejection and mixed chimaerism in patients that received previous ATG–CsA, compared with those that did not receive prior ATG–CsA (Table 1, $P < 0{\cdot}05$). The survival rate did not differ between patients who received ATG–CsA and those who did not (97·0% vs. 93·9%, respectively), but the probability of FFS at 8 years was 86·8 ± 6·2% in ATG–CsA recipients compared

Table 1. Comparison of characteristics of children with aplastic anaemia who underwent transplantation with and without prior ATG and CsA.

	ATG and CsA (n = 33)	No ATG and CsA (n = 33)	P-value
Gender (M/F)	16/17	15/18	NS
Age (years)	10 (0–15)	7 (0–18)	NS
Severity at transplantation: VS	8	14	NS
S	17	16	
Non-S	8	3	
Aetiology: idiopathic	32	32	NS
Hepatitis	1	1	
Diagnosis to transplantation (months)	9·6 (1–59)	2 (0–136)	NS
Transfusion: RC	8 (0–72)	6 (0–44)	<0·05
PC	10·5 (2–55)	8 (0–70)	NS
Conditioning			<0·05
CY + ATG/ALG	15	20	
Flu + CY + TLI/TAI	5	10	
Flu + CY + ATG/ALG	1	2	
CY + TLI	5	0	
Other	7	1	
GVHD prophylaxis			NS
MTX + CsA	27	30	
MTX + FK506	4	0	
CsA + mPSL	1	0	
CsA	1	3	
Infused cell counts ($\times 10^8$/kg)	4·0	3·7	NS
AGVHD (≥grade II)	2	1	NS
CGVHD	3	2	NS
Graft	30	33	NS
Survival	32	31	NS
Late rejection	7	2	NS
Mixed chimerism	12	4	<0·05
Re-transplantation	4	1	NS
Treatment effect (includes re-transplantation)			NS
CR	23	25	
PR	6	5	

VS, very severe; S, severe; Non-S, not severe; CY, cyclophosphamide; ATG, antithymocyte globulin; ALD, antilymphocyte globulin; Flu, fludarabine; TLI, total lymphoid irradiation: TAI, total abdominal irradiation; GVHD, graft-*versus*-host disease; aGVHD, acute GVHD; cGVHD, chronic GVHD; MTX, methotrexate; CsA, ciclosporin; FK506, tacrolimus; mPSL, methylprednisolone; CR, complete response; PR, partial response; NS, not significant; RC, red cell; PC, platelet concentration.

with 69·7 ± 8·0% for those that did not receive ATG–CsA (P = 0·044, Fig 1).

Discussion

Ades *et al* (2004) recently analysed risk factors for survival in 133 patients with severe AA who received BMT from HLA-identical siblings. In their study, the 10-year survival estimate was 64%. Due to progress in transplantation and supportive care over the last 2 decades, the survival rate has dramatically improved, from <60% to >90%. Multivariate analysis in the study by Ades *et al* (2004) showed that use of any form of specific treatment prior to BMT had a detrimental effect on overall survival rate. The kinds of specific treatments included androgen (n = 39), ATG (n = 32), and CsA (n = 17), which is of concern as the use of combined ATG–CsA therapy is currently a standard regimen in IST. In our study, use of a specific treatment for AA was not associated with an increased incidence of graft-rejection (P = 0·673), but the use of combined IST with ATG and CsA was a significant risk factor for graft-rejection (P = 0·044). The incidence of graft-rejection was the same between patients who did or did not receive G-CSF. In conclusion, our data suggest that the overall survival rate is excellent in patients with AA who receive BMT from HLA-identical siblings, but FFS is still unsatisfactory because of a high incidence of graft-rejection. Based on our results and the report from the Saint Louis Hospital (Ades *et al*, 2004), BMT should be instituted

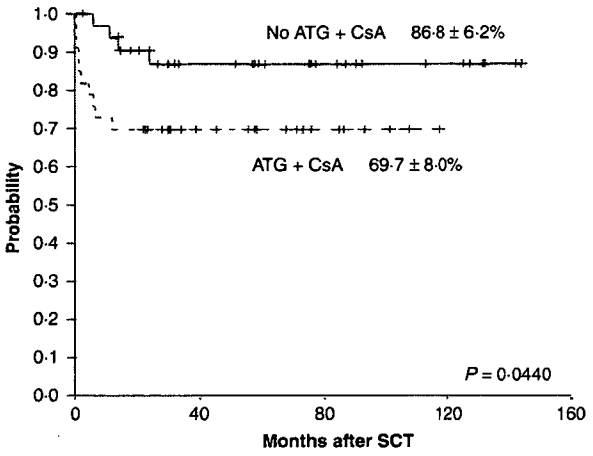

Fig 1. Kaplan–Meier estimate of failure-free survival for patients who did (ATG–CsA) and did not (no ATG–CsA) receive prior immunosuppressive therapy with ATG and CsA: ATG–CsA ($n = 33$): 69·7 ± 8·0%, no ATG-CsA ($n = 33$): 86·8 ± 6·2%, $P = 0·0440$. Failure-free survival was defined as survival with sustained engraftment, whereas death, graft-rejection and late graft-rejection were categorised as treatment failures.

immediately without IST in children with severe SAA who have an HLA-identical sibling.

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Perspective

Hsp90 and the Fanconi Anemia Pathway

A Molecular Link Between Protein Quality Control and the DNA Damage Response

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Hsp90, geldanamycin, Fanconi anemia, DNA repair, DNA damage, genomic instability, modifier gene, replicative stress, cancer chemotherapy, stem cell

ABSTRACT

Heat shock protein 90 (Hsp90) is a molecular chaperone that plays an essential role in cell growth and survival. The chaperone exerts these functions by regulating key signaling proteins involved in cell growth/survival and protecting cells from proteotoxic stress. Importantly, Hsp90 inhibitors including geldanamycin analogues show anti-tumor effects. We recently found that Hsp90 promotes stabilization and nuclear localization of the Fanconi anemia (FA) protein FANCA, which is required for activation of the FA pathway. The FA pathway is a multiprotein biochemical pathway involved in genotoxic signaling, defects in which cause genomic instability, hematopoietic stem cell failure and tumor development. Inhibition of Hsp90 impairs the intracellular homeostasis of FANCA, resulting in disruption of the FA pathway. These findings have important implications for rational cancer chemotherapy using Hsp90 inhibitors. We also discuss the possible functions of Hsp90 in FA pathophysiology and stem cell/cancer biology. Based on our findings and other data, we propose that Hsp90 functions as "a guardian of the genome" through the control of DNA repair proteins.

INTRODUCTION

Cellular responses to DNA damage are regulated by multiple distinct but highly interconnected pathways. In recent years, significant progress has been made in our understanding of the molecular mechanisms of genotoxic signaling. In this signaling pathway, DNA damage-induced activation of protein kinases (for example, ATM and ATR) and ubiquitin ligases (for example, Rad18) and consequent phosphorylation and ubiquitination of their substrates play an essential role in the recruitment of various DNA repair proteins present in the nucleus to the chromatin at sites of DNA damage, where they are assembled into multiprotein DNA repair machineries.[1-3] On the other hand, the amplitude of DNA damage responses is profoundly influenced by the nuclear levels of key DNA repair proteins, which are regulated by the balances between protein synthesis and degradation and between nuclear import and export. Prominent examples are seen in familial breast cancer-associated proteins such as BRCA1 and BRCA2. BRCA1 and its binding partner BARD1 shuttle between the cytoplasm and nucleus; and heterodimer complex formation by these proteins promotes their nuclear localization, potentially regulating their functions.[4] A recently identified BRCA2-interacting protein PALB2 is required for the stability and localization of BRCA2, with these two proteins functioning as essential partners.[5] Remarkably, the PALB2 gene was recently found to be responsible for familial susceptibility to breast cancer.[6,7]

MOLECULAR MECHANISMS OF THE FANCONI ANEMIA DNA DAMAGE RESPONSE PATHWAY

Fanconi anemia (FA) is a genetically heterogeneous inherited disorder characterized by congenital anomalies, progressive bone marrow failure and susceptibility to leukemia and solid tumors. Cells from FA patients are hypersensitive to DNA crosslinkers such as mitomycin C, showing chromatid breaks and apoptosis after exposure to such drugs at low concentrations. To date, 13 FA genes have been identified. The protein products encoded by these genes form a complicated biochemical pathway involved in the DNA damage response, which is closely connected with familial breast cancer-associated proteins.[8-11] This pathway, called "the FA pathway" or "the FA/BRCA pathway", includes three major stages

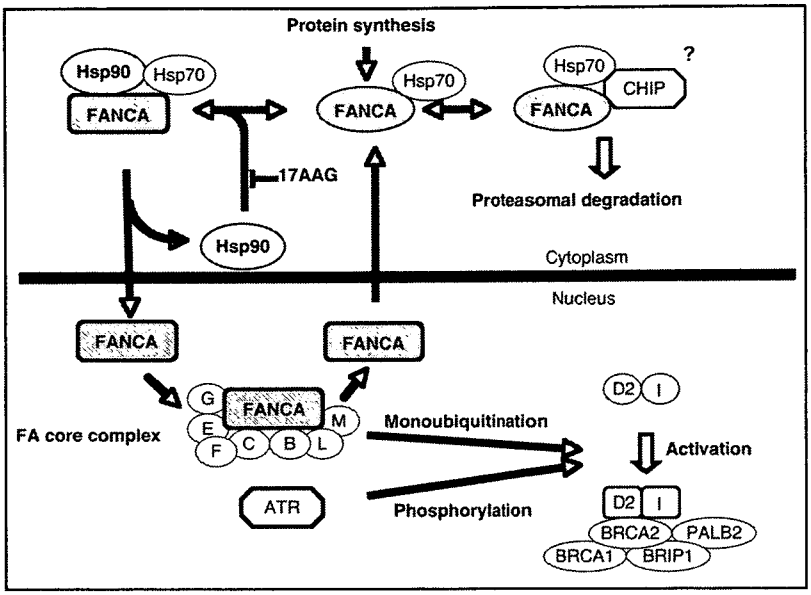

Figure 1. Molecular mechanisms of the FA pathway and the role of Hsp90. FANCA shuttles between the cytoplasm and the nucleus. The Hsp90-based multichaperone complex associates with cytoplasmic FANCA, newly synthesized and exported from the nucleus, promoting its folding into proper conformation required for stabilization and nuclear import. Hsp90 is probably recycled to form a complex with FANCA in the cytoplasm. 17-AAG-mediated inhibition of the chaperone cycle leads to proteasomal degradation of FANCA, possibly through Hsp70-mediated association with a chaperone-dependent ubiquitin-ligase CHIP. In the nucleus, FANCA, FANCB(B), FANCC(C), FANCE(E), FANCF(F), FANCG(G), FANCL(L) and FANCM(M) are assembled into a multisubunit complex (FA core complex) that is required for the downstream activation of the FANCD2(D2)/FANCI(I) complex. See text for other details.

(Fig. 1).First, at least eight FA proteins including FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL and FANCM are assembled into the FA 'core complex' in the nucleus. FANCL is an E3 ubiquitin-ligase.[8] Second, FANCD2 and its paralogue/binding-partner FANCI are activated by ATR-mediated phosphorylation and the FA core complex-dependent monoubiquitination.[8,9] Third, the active forms of FANCD2 and FANCI are targeted to the chromatin at sites of DNA damage, where they interact with DNA repair proteins including BRCA1 and BRCA2. Interestingly, BRCA2 is identical to FANCD1, and BRCA2-binding protein PALB2 is identical to FANCN.[10,11] Patients with biallelic mutations of these two FA genes (namely, type-D1 and type-N FA patients) characteristically show severe developmental anomalies and pediatric cancers.[8,11] BRCA2 and PALB2 play a critical role in homologous recombination-mediated DNA repair.[5] A BRCA1-associated DNA helicase BRIP1/BACH1 is responsible for type-J FA, but its function in the FA pathway is largely unknown.[8,12] Several studies have suggested that some FA proteins included in the core complex and FANCD2 are involved in the regulation of homology-directed DNA repair.[13,14] However, increasing evidence suggests that these FA proteins function to maintain genome stability through the coordination of multiple DNA repair mechanisms including homologous recombination, non-homologous end-joining and translesion synthesis.[8]

Although there is a notion that the FA core complex is formed in a constitutive manner, several lines of evidence suggests that post-translational control of stability, subcellular localization and protein modification of FA proteins influences function of the FA core complex. For instance, defects in the core complex components, FANCA, FANCC, FANCG and FANCF, seem relatively common in leukemic cells;[15] however, genetic abnormalities of the corresponding FA genes are rarely detected in sporadic cases of leukemia, despite extensive analysis.[16] Thus, aberrant stability control of FA proteins might be involved in the pathogenesis of leukemia. FANCA is primarily localized in the nucleus but shuttles between the cytoplasm and the nucleus.[17,18] FANCA is imported into the nucleus using a bipartite nuclear localization signal (NLS) in its N-terminal region, and is exported from the nucleus through a CRM1-dependent nuclear export machinery.[17,18] FANCG directly binds to the N-terminal region containing the NLS and stabilizes FANCA.[19] Interestingly, overexpression of FANCG not only elevates FANCA protein levels but also prevents nuclear localization of FANCA probably by masking the NLS (Oda T, Yamashita T, unpublished data). In addition, nuclear localization of FANCA seems to be profoundly influenced by its conformation in the C-terminal region, since various patient-derived mutations in this region prevent the nuclear import of FANCA.[20] Furthermore, FANCA is phosphorylated at an unknown serine residue(s), and its phosphorylation level correlates with nuclear localization.[21,22] Finally, impaired activation of the FA pathway due to mislocalization of FANCA was observed in a patient with acquired bone marrow failure.[23] Taken together, these observations suggest that the FA pathway is affected by post-translational regulation of the turnover and trafficking of FANCA. However, the underlying molecular mechanisms remain largely unknown.

We recently reported that the molecular chaperone Hsp90 regulates the FA pathway.[24] During analysis of the FANCA-containing protein complex, we identified Hsp90 and Hsp70, which often cooperate in the form of a multichaperone complex, as possible FANCA-associated proteins. Geldanamycin and its analogues such as 17-allylamino-17-demethoxygeldanamycin (17-AAG) specifically inhibit the chaperone activity of Hsp90 through masking its ATP-binding domain, thus providing a powerful tool with which to study the function of this chaperone. We demonstrated that a cytoplasmic fraction of FANCA interacts with Hsp90 in a 17-AAG-sensitive manner. Furthermore, treatment of cells with 17-AAG induces rapid proteasomal degradation and cytoplasmic retention of FANCA. These data strongly suggest that an Hsp90-based chaperone machinery has a critical role in the stabilization and nuclear import of cytoplasmic FANCA, and that inhibition of Hsp90 induces nuclear depletion of the FA core complex, leading to impaired activation of the FA pathway. Indeed, 17-AAG inhibits DNA damage-induced activation of FANCD2 and enhances DNA crosslinker-induced cytotoxicity, but this drug effect is much less pronounced in FA pathway-defective cells. Furthermore, 17-AAG markedly enhances DNA crosslinker-induced chromosome aberrations. On the other hand, 17-AAG has little effect on ATR activation. Based on these results, we concluded that Hsp90 promotes activation

of the FA pathway through the regulation of FANCA homeostasis.[24] To further confirm this notion, our preliminary data indicate that RNAi-mediated depletion of Hsp90 causes cytoplasmic retention of FANCA (Sekimoto T, Yamashita T, unpublished data).

Hsp90 AND DNA DAMAGE TOLERANCE

Hsp90 and Hsp70 are molecular chaperones that function as part of a protein quality control system.[25,26] They promote not only the maturation of newly synthesized proteins into functional forms, but also the refolding, disaggregation and proteasomal degradation of denatured proteins. In particular, Hsp90 regulates the function and stability of various signaling proteins which play key roles in the regulation of cell growth/survival.[26] These include nuclear hormone receptors, protein kinases (for example, ErbB2 and Raf-1) and transcription factors (for example, p53 and HIF-1α). Importantly, Hsp90-inhibiting geldanamycin derivatives are currently in clinical trials as promising cancer chemotherapeutic agents.[27] The anti-tumor effect of Hsp90 inhibitors is mainly attributed to the proteasomal degradation of cell growth/survival signaling proteins.[26,27] Our findings provide new insights into FA pathophysiology, pharmacological effects of Hsp90 inhibitors and the regulatory mechanisms of cellular DNA damage tolerance.

One interesting hypothesis arising from our findings is that Hsp90 may affect a genotype-phenotype correlation in FA. Extensive mutational analyses in various genetic diseases have revealed that the molecular basis of genotype-phenotype relationship is complicated even in Mendelian inherited disorders. In most of these 'single-gene' diseases, patients with identical mutations in a responsible gene show a broad spectrum of clinical manifestations. Such phenotypic variation is partly explained by modifier genes.[28] Importantly, identification of such modifiers may be useful for developing preventive/therapeutic tools for treating genetic diseases. Although phenotypic variability of FA is partly explained by genetic heterogeneity[11,29] and reversion mosaicism,[30,31] unidentified modifier genes are likely to be involved. Our results raise the possibility that Hsp90 functions as a modifier in FA. To support this notion, Hsp90 is known to mask the phenotypic consequences of mutant proteins by assisting their folding in plants and flies, whereas these cryptic phenotypes are uncovered when Hsp90 functions are compromised by environmental stress.[32] Since FANCA is a polymorphic protein, it is tempting to speculate that Hsp90-assisted folding helps FANCA mutant proteins exert normal or subnormal functions. In this context, environmental stress overloading Hsp90 might affect the clinical phenotypes of FA patients.

Our findings reveal a novel pharmacological effect of Hsp90 inhibitors, which has important implications for the rational design of cancer chemotherapies using Hsp90 inhibitors. DNA interstrand crosslinkers including bifunctional alkylating agents and platinum-based drugs are a major class of anti-tumor agents.[33] Growing evidence suggests that the activity of the FA pathway is an important determinant for tumor sensitivity to these drugs. Epigenetic inactivation of the FANCF gene in several tumor types is associated with their hypersensitivity to DNA crosslinkers.[34] On the other hand, enhanced activity of the FA pathway seems to be responsible for DNA crosslinker resistance.[35] In addition, pharmacological inhibitors of the FA pathway could be used as chemosensitizers of tumor cells to these drugs. For example, curcumin inhibits the FA pathway by unknown mechanisms and sensitizes tumor cells to cisplatin.[36] Similarly, combined use of Hsp90 inhibitors and DNA crosslinkers may have synergistic anti-cancer effects. However, our results raise the possibility that Hsp90 inhibitors cause acquired FA-like disorders as a result of inactivation of the FA pathway, especially when they are used long-term and/or repeatedly.

Hsp90-mediated potentiation of the FA pathway may be important for tumor cell survival in physiological situations. Tumor cells typically show increased expression and activity of Hsp90, which is generally believed to contribute to tumor cells' tolerance to various proteotoxic stressors derived from the microenvironment, including hypoxia and acidosis.[26] On the other hand, tumor cells seem to suffer from continuous replicative stress, derived from their aberrant proliferation, oxidative DNA damage and possibly deprivation of nutrients required for DNA synthesis.[37] These observations suggest that tolerance against replicative stress is probably an important factor for survival advantage of tumor cells. The FA pathway seems to play an integral role in the cellular response to replicative stress through coordinating enzymatic DNA processing, homologous recombination, translesion synthesis and activation of cell cycle checkpoints.[8,38] Thus, we postulate that Hsp90 promotes tumor cell tolerance to replicative stress through activation of the FA pathway, contributing to tumor cell survival. It is plausible that a similar mechanism is involved in the survival of adult stem cells in normal tissues, given that normal stem cells and tumor cells share a number of molecular machineries for their growth/survival.

CONCLUSION

The FA pathway plays an essential role in protecting the genome from genotoxic stress, which is critical for the maintenance of adult stem cells and tumor suppression, especially in hematopoietic tissues. This mechanism is utilized for DNA damage tolerance in tumor cells. We have established that Hsp90-mediated regulation of FANCA has a profound impact on the FA pathway. In addition, Hsp90 regulates various proteins involved in genomic stability, including telomerase, Chk1, topoisomerase II and the NBS1-Mre11-Rad50 complex.[39-42] We propose that Hsp90 functions as a guardian of the genome through the regulation of several DNA damage response pathways, thus playing an important role in the growth and survival of normal stem cells as well as tumor cells.

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A requirement of FancL and FancD2 monoubiquitination in DNA repair

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The rare hereditary disorder Fanconi anemia (FA) can be caused by mutations in components of the FA core complex (FancA/B/C/E/F/G/L/M), a key regulator FancD2, the breast cancer susceptibility protein BRCA2/FancD1, or the newly identified FancJ/BRIP1 helicase. By performing yeast two-hybrid (Y2H) screens using N-terminal chicken (ch) FancD2 as a bait, we have identified chFancL, the likely ubiquitin E3 ligase subunit of the FA core complex. We also found that ectopically expressed FancD2 and FancL co-immunoprecipitated in 293T cells, and this interaction was dependent on the PHD domain of FancL. *FANCL*-disrupted chicken DT40 cells displayed defects in both FancD2 monoubiquitination and focus formation. Importantly, cell lines lacking the *FANCL* or *FANCD2* genes, or carrying a "knock-in" mutation of the FancD2 monoubiquitination site (where the Lys 563 residue is changed to Arg), displayed quantitatively identical defects in the repair of I-*Sce*I-induced chromosomal breaks by homologous recombination (HR). These data establish the role of *FANCL* and FancD2 monoubiquitination in HR repair.

Introduction

Fanconi anemia (FA) is a rare hereditary disorder characterized by bone marrow failure, compromised genome stability and an increased incidence of cancer (D'Andrea 2003; Venkitaraman 2004). Complementation analysis revealed at least 12 causative genes, and 11 of them (FancA/B/C/D1/D2/E/F/G/J/L/M) have been cloned (Thompson 2005). Cells lacking these genes display increased levels of chromosome breakage, particularly following induction of DNA interstrand cross-links (ICL) by drugs such as mitomycin C (MMC) or cisplatin (Sasaki & Tonomura 1973; Sasaki 1975). This property has been used as a diagnostic hallmark for FA, and suggests an important role for FA proteins in repairing ICLs. The molecular mechanism of ICL repair (McHugh *et al.* 2000; Dronkert & Kanaar 2001) is still poorly understood; however, it likely requires the intimate interplay of a number of distinct repair pathways and molecules (Nojima *et al.* 2005), including nucleotide excision repair, homologous recombination (HR) repair, translesion DNA synthesis (TLS), SNM1A (Sensitivity to Nitrogen Mustard 1A) and SNM1B (Ishiai *et al.* 2004) and FA proteins.

Recent studies have indicated that the FA pathway promotes DNA repair function through both HR and TLS pathways (Yamamoto *et al.* 2003; Niedzwiedz *et al.* 2004; Hirano *et al.* 2005; Nakanishi *et al.* 2005). However, how FA proteins exert their function is still unclear. Eight FA proteins constitute the nuclear FA core complex (FancA/B/C/E/F/G/L/M). Only two of them, FancL and FancM, have conserved sequence motifs that suggest an obvious catalytic function. FancL contains a

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PHD-type zinc-finger (PHD finger) domain (Meetei *et al.* 2003a), while helicase and nuclease domains are found in the FancM protein (Meetei *et al.* 2005; Mosedale *et al.* 2005), which is homologous to Archea Hef (Nishino *et al.* 2005). Upon DNA damage or replication fork stalling, the FancD2 protein is modified by monoubiquitination on a specific Lys residue (Lys 561 in human (h) protein). Following monoubiquitination, which is likely mediated by FancL (Meetei *et al.* 2003a), FancD2 is targeted to chromatin and accumulates at or close to sites of damaged DNA, forming nuclear foci that can be detected by immunofluorescence. Consistent with the functional studies (Nakanishi *et al.* 2005; Yamamoto *et al.* 2005), FancD2 foci co-localize with HR proteins, such as Rad51, BRCA1 (Taniguchi *et al.* 2002a) and BRCA2 (Wang *et al.* 2004), or with the TLS factor Rev1 (Niedzwiedz *et al.* 2004) following ICL induction. This monoubiquitination seems both necessary and sufficient for FancD2 activation as shown by ectopic expression of either mutated FancD2 (Garcia-Higuera *et al.* 2001; Taniguchi *et al.* 2002b) or a FancD2-ubiquitin fusion protein (Matsushita *et al.* 2005). Any mutation that has been identified in the core complex components appears to compromise the E3 ligase activity as well as structural integrity of the complex, resulting in common down-stream defects in monoubiquitination and focus formation of FancD2. In addition, the disrupted core complex may lose its own effector function, which is still undefined but might be carried out by components such as FancM (Matsushita *et al.* 2005; Meetei *et al.* 2005; Mosedale *et al.* 2005).

Identification of the other two FA genes, FancD1/BRCA2 (Howlett *et al.* 2002) and FancJ/BRIP1 (Bridge *et al.* 2005; Levitus *et al.* 2005; Levran *et al.* 2005; Litman *et al.* 2005), has forged a strong link between FA and familial breast cancer. While mutations in a single allele of BRCA2 predispose carriers to breast or ovarian cancer, biallelic hypomorphic mutations are found in a subset of FA patients (Howlett *et al.* 2002). Such patients are clinically rather distinct, for they often develop early onset leukaemia and brain tumors, which are rarely seen in individuals from the other complementation groups (Hirsch *et al.* 2003; Wagner *et al.* 2004). FancJ/BRIP1 was originally identified through interaction with another breast cancer suppressor, BRCA1 (Cantor *et al.* 2001). The role of BRCA2 is well characterized as a major regulator of Rad51, a central molecule in HR that mediates homology searching and strand transfer (Venkitaraman 2002). On the other hand, BRIP1 protein has a helicase activity, which preferentially unwinds a forked duplex structure that may mimic a stalled replication fork (Gupta *et al.* 2005). Of importance, in both *fancd1* and *fancj* cell lines, monoubiquitination of FancD2 occurs normally, placing these molecules down-stream of, or parallel to, the FA core complex–FancD2 pathway (Howlett *et al.* 2002; Levitus *et al.* 2004).

In this study, we have identified chicken FancL as a FancD2-interacting protein by yeast two-hybrid (Y2H) screening. We have established and characterized a *FANCL*-deficient mutant DT40 cell line, and confirmed a role for FancL in HR. To rigorously test whether the physiological function of FancD2 totally relies on its monoubiquitination, we generated DT40 cells carrying a monoubiquitination site "knock-in" mutation (Lys 563 changed to Arg) at the endogenous *FANCD2* locus, and compared phenotypes among *FANCD2*-null mutant (designated *fancd2-null*), the "knock-in" K563R mutant (*fancd2-K563R*) and *FANCL*-deficient (*fancl*) cells. Our results establish a requirement for FancL and FancD2 monoubiquitination in the repair of DSBs mediated by HR.

Results

Identification of FancL as a FancD2-interacting protein by Y2H screening

Previous studies showed that FancD2 plays crucial roles in the DNA damage response; however, the precise molecular mechanism by which FancD2 is activated is largely unknown. To analyze this, we employed the Y2H screening to search for proteins that interact with FancD2, using an N-terminal chFancD2 fragment (chFancD2 2-722) (Fig. 2A). Full length chFancD2 was not suitable for screening because of autoactivation of the reporters (data not shown). We isolated 15 positive clones. DNA sequencing of the interacting clones revealed that one of them encoded full-length chPHF9, later identified as FancL (Meetei *et al.* 2003a). Chicken PHF9/FancL encodes a protein with 373 amino acids, and the percentage amino acid sequence identity with hFancL is 68%. Human FancL has three potential WD40 repeats and a PHD finger motif (Meetei *et al.* 2003a), and these were all well conserved in chFancL (NCBI/EMBL/DDBJ accession number: AB214907 for chFancL). A part of the amino acid sequence has been described in Matsushita *et al.* (2005) as supplement Fig. S4A.

To confirm the interaction detected by Y2H, we transfected 293T cells with vectors which express FLAG-tagged chFancD2 and GFP-tagged chFancL. In anti-FLAG immunoprecipitates prepared from cell lysates of the transfected 293T cells, anti-GFP Western blotting could successfully detect the presence of GFP-chFancL (Fig. 1). Furthermore, we detected Y2H interaction between N-terminal fragment of hFancD2 (1-719 amino acids) and hFancL (Table 1), indicating that FancD2–FancL interaction is evolutionarily conserved.

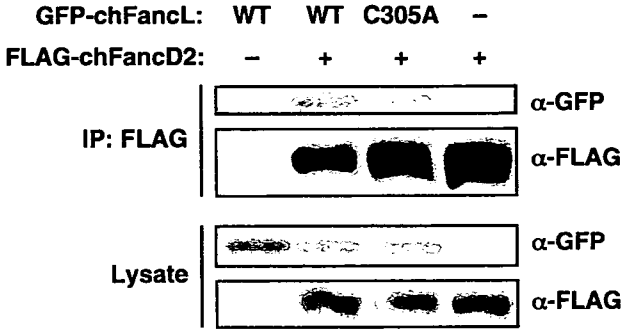

Figure 1 Co-immunoprecipiation of FLAG-chFancD2 with GFP-chFancL. 293T cells were co-transfected with FLAG-chFancD2 and GFP-chFancL, and lysates were prepared 24 h after transfection. WT or C305A, wild-type or the PHD domain mutant (C305A) of chicken FancL, respectively. After anti-FLAG immunoprecipitates or whole cell lysates were separated by SDS-PAGE, GFP-chFancL was detected by Western blotting using anti-GFP antibody.

We extended the Y2H assay to determine which regions were important for the interaction between chFancD2 and chFancL. First, we focused on chFancD2. To cover the entire chFancD2 protein (1439 amino acids), we made three bait plasmids each carrying different chFancD2 fragments (residues 2-429, 162-1082 and 1083-1439), but none of these could interact with full-length chFancL using the Y2H assay (Fig. 2A). Hence, the original bait used for screening (chFancD2 2-722) was divided into three pieces; namely 2-177, 162-429 and 430-722, and these were similarly tested for Y2H interaction with chFancL. Again, none of the fragments could bind to chFancL (data not shown). These data suggest either that a large portion within chFancD2 2-722 is required for interaction with chFancL or that the interaction domain is formed from two or more non-contiguous regions of the polypeptide. Note that the bait chFancD2 2-722 contains the Lys 563 monoubiquitination site. However, the similar bait carrying K563R mutation (chFancD2 KR 1-758) was still able to interact with chFancL (data not shown).

Next, we tried to narrow down the interacting portion in chFancL. To test whether the PHD domain is important for FancL–FancD2 interaction, we changed the two conserved amino acid residues to Ala (chFancL C305A, which was described in Matsushita *et al.* (2005), and W339A). In addition, the Pro 330 and the Gln 348 residues, which are conserved from human to fish, were each mutated to Lys and Thr (P330K and Q348T), the corresponding residues found in the *Drosophila* FancL. The C305A mutation reduced the chFancD2–chFancL interaction in both Y2H (Fig. 2C) and co-immunoprecipitation

Table 1 Interactions between human FancL, various FA genes by Y2H

	Bait: human FA genes						
Prey	A	B	C	D2	E	F	G
hFancL	+*	–	–	+	–	+	N.T.†
hFancLC307A	–	–	–	–	–	–	N.T.
vector	–	–	–	–	–	–	N.T.

+, weak growth and pale blue colonies; –, no growth and white colonies.
*Activation of *LEU2* and *β-galactosidase* (*lacZ*) reporter genes as shown in Fig. 2.
†N.T., not testable because of the autoactivation of reporters.

(Fig. 1) assays. This could be due to destruction of the whole protein structure, since the equivalent C307A mutation in hFancL impaired the interaction with a few other FA proteins (Table 1, see below). However, the other PHD domain mutations (P330K and Q348T), but not W339A, also affected the interaction (Fig. 2B,C) indicating that the PHD finger domain is required for interaction with chFancD2. We also divided the full length chFancL cDNA into four overlapping fragments as shown in Fig. 2B, and tested Y2H interaction. None of them interacted significantly with the chFancD2 2-722 bait, indicating that both PHD domain (305-374 region) and N-terminal 1-143 region are required for the interaction. The inverse combination of the bait–prey was not feasible, since the bait contained the full-length chFancL autoactivated reporters (data not shown).

Since hFancL was isolated as a FancA-associated protein, it should be a part of the FA core complex (Meetei *et al.* 2003a). We examined whether FancL directly binds to other components of the FA core complex by Y2H. The bait plasmids were prepared by subcloning a variety of human FA core complex genes, because fewer chicken FA genes were available. Using hFancL as a prey, we found that, besides hFancD2, only hFancA and hFancF displayed significant binding with hFancL (Table 1). These interactions were disrupted by the C307A mutation (equivalent to chFancL C305A). Consistently, a previous report showed that co-immunoprecipitation between hFancA and hFancL was reduced by the C307A mutation (Meetei *et al.* 2003a).

FANCL-deficient cells show defects in FancD2 activation and repair of DNA damage

To characterize the physiological function of FancL, we generated *FANCL* gene disruptants in the chicken B cell