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S-phase fraction as a useful marker for prognosis and therapeutic response in patients with aplastic anemia

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BACKGROUND: The functional definition of aplastic anemia (AA) is the failure of hematopoietic stem cells to proliferate. The aim of the present study was to analyze the S-phase fraction (SPF) (proliferative activity) in patients with AA at diagnosis to explore its relationship with disease characteristics and its value in discriminating among patients with different prognoses. We also investigated whether the SPF value influenced the response to immunosuppressive therapy in AA patients.

PATIENTS AND METHODS: The analysis of SPF at the time of diagnosis was carried out by flow cytometry on peripheral blood samples from 53 consecutive patients with AA and 30 age- and sex-matched controls. All patients were given cyclosporine and followed up periodically to determine response to therapy.

RESULTS: Based on the median SPF, AA patients were divided into two groups: patients with SPF ≤0.59% (n=27) and patients with SPF >0.59% (n=26). An SPF >0.59% was associated with advanced age (*P*=.02) and elevated serum LDH level (*P*=.01). Patients with an SPF >0.59% also had a higher incidence of paroxysmal nocturnal hemoglobinuria and cytogenetic abnormalities. During a median follow-up of 18 months, 3.7% of patients with SPF ≤0.59 and 11.5% of patients with SPF >0.59% developed dysplasia and one patient with SPF >0.59% converted into AML. A significantly higher (*P*=.018) overall response rate of 53.9% was found in patients with SPF >0.59% versus 22.2% of patients with SPF ≤0.59% at 6 months.

CONCLUSIONS: Independently of the peripheral blood count, the SPF at diagnosis may provide information on the expected response to immunosuppressive therapy and the propensity for disease to evolve into MDS/AML. Hence, SPF may serve as an early indicator for the evolution of MDS/AML in patients with AA and thus contribute to therapeutic decisions.

Aplastic anemia (AA) is a potentially life-threatening failure of hematopoiesis characterized by pancytopenia and bone marrow aplasia.[1] Patients with aplastic anemia have a stem cell defect both in proliferation and differentiation as shown by long-term bone marrow culture, long-term cell assays and committed progenitor assays.[2] Patients with AA have an increased risk of developing clonal hematological diseases including paroxysmal nocturnal hemoglobinuria (PNH), myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML).[3,4] The evolution from AA to MDS/AML is marked with increased proliferative activity with or without development of cytogenetic abnormalities. Around 10% to 20% of survivors of AA develop a clonal hematological disease within the decade following immunosuppressive therapy.[4,5] It is uncertain whether the development of secondary MDS or acute leukemia simply discloses the natural history of AA as a premalignant disease or whether it is related to the therapy applied.[5] There is a possibility that a proportion of cases of AA may be in fact cases with preleukemia.[6]

Cellular proliferation is an important prognostic parameter in cancers. Failure of proliferation, in the hematopoietic stem cells of AA patients is well known, whereas there is proliferation but with abnormal and minimal or no differentiation in cases of MDS/AML. The measurement of dividing cell fraction in S-phase by means of flow cytometry is an established method

for assessing proliferative activity. Histograms obtained by flow cytometry provide information related to neoplastic progression, i.e. SPF and ploidy. SPF measures the percentage of a given cycling subpopulation between G0/G1 and G2/M phase and represents a continuous variable related to any proliferating cell population.[7] SPF status has been studied in different hematological disorders including MDS, chronic myeloid leukemia, acute lymphoblastic leukemia, non-Hodgkin lymphomas and solid tumors such as prostate cancer and breast cancer for the purpose of prognostic classification.[7-14]

Determining the prognosis of patients with AA requires a careful assessment of conventional clinicopathologic parameters including dysplastic changes in peripheral blood, percentage of blasts in bone marrow and cytogenetic abnormalities.[15-18] However, these conventional factors have limited value in predicting the prognosis in individual patients; additional parameters are needed to achieve this goal. Reliable biomarkers are still lacking, and therefore, the clinical outcome is difficult to predict. The aim of the present work was to analyze the proliferation rate of peripheral blood cells using a standard and reproducible method (i.e., flow cytometry) to measure SPF to explore the relationship with disease characteristics and the value of SPF in predicting prognosis. We also investigated whether the SPF value influenced the response to immunosuppressive therapy in patients with AA.

PATIENTS AND METHODS

The study group comprised of 53 consecutive patients with AA and 30 age- and sex-matched healthy subjects. The diagnosis of AA was made on the basis of complete blood count (CBC), bone marrow aspiration, and biopsy. The severity of AA was defined according to standard criteria. Patients who had received anti-lymphocyte globulin/anti-thymocyte globulin, steroids or other immunosuppressive therapy were excluded from the study. All AA patients were given cyclosporine (5-10 mg/kg/day) as no patient was able to afford ATG/ALG or bone marrow transplantation (BMT). The patients were followed-up periodically for the response to treatment and side effects to the medication. The dosage of cyclosporine was adjusted according to clinico-hematological parameters. Investigations were performed at the time of diagnosis and prior to treatment. The SPF analysis and bone marrow examinations (aspiration and biopsy) were done at 6-month intervals whereas cytogenetic analysis was performed yearly. All patients with AA were also evaluated for the presence of PNH using the flow cytometry and sucrose lysis test at diagnosis and during follow up. Informed written consent was obtained from patients and control subjects. The study protocol was approved by the institutional ethics committee.

Flow cytometric analysis. SPF analysis (cell cycle analysis) was carried out according to the Krishnan method.[19] Peripheral blood was collected in a sodium heparin Vacutainer vial and 100 µL of heparinized peripheral venous blood was added to 1 mL of propidium iodide solution (50 µg/mL in 0.1% hypotonic solution of sodium citrate) and incubated for 10 min at 40°C. A 10 minute staining time at 40°C proved to provide the best histogram resolution. Samples were analyzed on a Becton Dickinson flow cytometer with Mod Fit-LT V 3.0 software using an excitation wavelength of 488 mm. Cells acquired on flow cytometry were plotted in side-scattered light versus forward-scattered light, fluorescence-2 area (FL-2A) versus fluorescence-2 width (FL-2W) and FL-2A versus count. The threshold was adjusted to have a first peak at the 200 channel of the FL-2W plot and the lymphocyte cell population was gated from a plot of FL-2A versus FL-2W for analysis. Gated lymphocytes were analyzed using a broadened rectangular model of Mod FIT-LT software with a constant coefficient of variation <6 and a residual chi-square <5. This model was also fitted with single-cut debris subtraction and aggregate subtraction. Ten thousand cells were analyzed per sample. Human peripheral blood lymphocytes from hematologically normal subjects were used as a diploid reference standard.

Response criteria. Complete response was defined as transfusion independence associated with hemoglobin (Hb) >11 g/dL, an absolute neutrophil count (ANC) $>1.5\times10^9$/L and a platelet count $>100\times10^9$/L. Partial response was defined as transfusion independence associated with Hb >8 g/dL, ANC $>0.5\times10^9$/L and a platelet count $>30\times10^9$/L. Transfusion dependence was taken as evidence of no response.[20]

Statistical analysis. The findings were expressed as the mean and standard deviation. The differences between the groups were compared with the use of the *t* test. The chi-square test was used when appropriate. A value of $P\leq.05$ was considered statistically significant.

RESULTS

Fifty-three consecutive patients with AA were included in the present study. Mean (SD) for the age of the patients with AA was 29.0 (15.5) years (n=53, range 13-72 years with a median age of 22 years) while that of control subjects was 30.5 (15.3) years (n=32, range 14-67 years with a median age of 25 years). The male-to-female ratio of patients with AA was 3.1:1. There were 13 patients (24.5%) with very severe aplastic anemia, 18 patients (34%) with severe aplastic anemia and 22 patients

Table 1. Cell cycle distribution of peripheral blood lymphocytes in control and aplastic anemia patients.

Cell cycle phase	Control (n=32)	Aplastic anemia (n=53)
G0/G1 phase (%)	98.5 (0.33)	98.71 (1.09)
S phase (%)	0.89 (0.15)	0.87 (0.85)
G2/M phase (%)	0.58 (0.20)	0.42 (0.29)
Coefficient of variation (%)	3.24 (1.12)	3.47 (1.05)

Data are mean and standard deviation.

Table 2. Relationship between the clinico-biological characteristics and S-phase fraction in aplastic anemia patients at diagnosis.

Characteristics	SPF ≤0.59% (n=27)	SPF >0.59% (n=26)	*P* value
Hemoglobin (g/dL)	4.52 (1.91)	5.24 (1.82)	NS
WBC count (×10^9/L)	2.46 (0.84)	2.78 (0.89)	NS
Platelet count (×10^9/L)	0.46 (0.22)	0.44 (0.20)	NS
Absolute neutrophil count (×10^9/L)	0.57 (0.53)	0.83 (0.60)	NS
Male, n (%) Female, n (%)	21(77.8) 6 (22.2)	18 (69.2) 8 (30.8)	NS
Age	24.26 (11.8)	33.52 (17.5)	.02
Lactate dehydrogenase (IU/L)	323.4 (118.5)	439.9 (189.1)	.01

Data are mean and standard deviation, unless noted otherwise.

Table 3. S-phase fraction (SPF) value and response to immunosuppressive therapy (cyclosporine) at 6 months.

	SPF ≤0.59% (n=27)	SPF >0.59% (n=26)
Complete response, n (%)	1 (3.7)	3 (11.5)
Partial response, n (%)	5 (18.6)	11 (42.3)
Overall response, n (%)*	6 (22.2)	14 (53.9)

Overall response: complete response + partial response * *P*=.018.

(41.5%) with non-severe aplastic anemia. The cause of AA was not obvious in 88.7% of the cases. A possible association of NSAIDs with the disease was found in 11.3% cases. The most common clinical presentations were dyspnea on exertion, fatigue and pallor. There were no recurrent infections, but in 4 cases (7.5%) mouth ulcer was present at diagnosis. Gum bleeding was present in 5 cases (9.4%).

Cell cycle analysis on the peripheral blood of patients with AA at the time of diagnosis showed that most of the cells were in G0/G1 phase (Table 1). The median value of SPF in patients with AA was 0.59% with a range of 0.06% to 3.47%, which was lower than that of controls (median value 0.90%, range 0.54% to 1.16%). There were 8 patients who had an SPF value higher than the upper limit SPF value found in controls. In these patients, the mean SPF value was 2.69±0.75% with a range of 1.57% to 3.47%. On the basis of the median SPF, patients were divided into two groups whose clinical and hematological characteristics are shown in Table 2. Patients with SPF >0.59% had a higher mean age (*P*=.02) and serum lactate dehydrogenase (*P*=.01). than the patients with SPF≤0.59%. Patients with SPF >0.59% also had higher incidence of PNH (15.4% versus 7.4% in the SPF ≤0.59% group). Chromosomal abnormalities in the form of trisomy 8 and trisomy 21 were present in 2 patients (3.77%) with AA at the time of diagnosis. Both patients had SPF >0.59%. The median follow-up of patients was 18 months (range, 6 to 24 months). During the follow-up, 3.7% and 11.5% patients developed dysplasia in the groups with SPF ≤0.59% and >0.59%, respectively. One patient with SPF >0.59% converted into AML. Half of the patients who had a higher SPF value than the upper limit SPF value of controls at diagnosis developed dysplasia/AML during the follow-up. Cytogenetic abnormalities developed during the follow-up in five more cases. These abnormalities were trisomy 8 (two patients), trisomy 6 (two patients) and monosomy 7 (one patient). The incidence of cytogenetic abnormalities was higher (19.2%) in patients with SPF >0.59% than in patients with SPF ≤0.59%. The patients with SPF >0.59% had a better response to immunosuppressive therapy (cyclosporine) that was statistically significant (*P*=.018) (Table 3). Fourteen patients died during the study; most (64.3%) were in the SPF ≤0.59% who also did not respond to therapy.

DISCUSSION

AA reflects the failure of proliferation of hematopoietic stem cells in bone marrow manifesting as a decrease in blood cell counts. The mechanisms suggested for bone marrow failure are: a) direct hematopoietic injury by chemicals, drugs or radiation to both proliferating and quiescent hematopoietic cells, and b) immune-mediated suppression of marrow cells.[21] Therefore, patients with AA have very low proliferative rates, which rise from low to normal as patients respond to immunosuppressive therapy. In a prospective, randomized, multicenter study of immunosuppressive therapy in patients with AA, a significantly higher overall response rate of 74% was found in ATG plus cyclosporine groups whereas the response to cyclosporine alone was 46% at 6 months.[22] However, the response to immunosuppressive therapy

in AA is quite variable (40% to 70%).[23] The reason for this is not well known though it may depend on the age of patients, severity of the disease, presence of PNH and cytogenetic abnormalities.[24-27] The occurrence of PNH in patients with AA is well known and may arise with disease progression. In various studies, 10% to 57% patients of AA developed a PNH clone during long-term survival.[28] The 10-year cumulative incidence rate of MDS and AML in patients with AA responding to immunosuppressive therapy was 9.6% and 6.6%, respectively.[29] It was not clear whether the transformation was a natural course of the disease or due to immunosuppressive therapy. Furthermore, patients with AA having cytogenetic abnormalities are more prone to develop MDS and AML.[17,18]

It is likely that some patients diagnosed with AA actually have hypoplastic myelodysplastic syndrome (HMDS) that is not apparent enough to be diagnosed by current methods. Attempts have been made to assess the proliferative behavior of hematopoietic cells in patients with AA to find evidence of dysplasia and thus to make a prognostic evaluation and therapeutic decisions. AA patients with dysplasia (HMDS) should be treated with BMT rather than immunosuppressive therapy.[30] In recent years, hematologist and pathologists have widely applied immunohistochemistry techniques using antigens (proliferating cell nuclear antigen, CD34 and p53) and mitotic activity on chromosome preparations to assess the proliferative rate, but these are not reproducible and satisfactory.[31-34] Flow cytometry measurement of cells in S-phase is a validated method for measuring cell proliferation. SPF has been studied in a number of hematological diseases and solid tumors for the purpose of classification, grading and prognosis.[7-14] Its advantage is that it can be used on a wide variety of tissue preparations, including peripheral blood, frozen biopsy specimens, fresh surgical samples and archival paraffin blocks.

There are a few studies on the cell cycle and proliferative behavior of cells in patients with AA, but none on SPF to our knowledge. We found that the SPF value in patients with AA was quite variable (range 0.06% to 3.47%) and that the median value of SPF (0.59%) was lower than that in controls (median 0.90%, range 0.54% to 1.16%). This is conceivable since AA is characterized by limited proliferation of hematopoietic stem cells. In view of the wide variability in SPF in patients with AA, we classified patients into two groups based on the median value. Our data showed that the SPF rises with advanced age in patients with AA. Cytogenetic abnormalities were present in a higher proportion of patients with SPF >0.59% as compared to patients with SPF ≤0.59%, which is supported by published reports that older AA patients are more likely to develop cytogenetic abnormalities, dysplasia or AML.[24] Although the majority of older patients, even those with SPF >0.59% did not show cytogenetic abnormalities, it is likely that they may be harboring dysplasia in the bone marrow that is not apparent morphologically. In such cases, SPF may be an indicator for evolving dysplasia in the bone marrow.

We showed that during the follow-up, 3.7% of patients SPF ≤0.59% and 11.5% of patients with SPF >0.59% developed dysplasia. One patient converted into AML who belonged to SPF >0.59% whereas none of patients with SPF ≤0.59% converted into AML. We found that patients with a higher SPF value than the upper limit of SPF of controls at diagnosis were more prone to develop dysplasia/AML. This observation is in agreement with other reports on various hematological disorders as well as solid tumors.[7-14] A serial measurement of SPF in patients with AA may provide an early indication for development of dysplasia. The lower SPF value (≤0.59%) was associated with a poor response in our study. It is likely that patients with a lower SPF (≤0.59%) have a stem cell or precursor defect and thus show poor response. The proliferative deficit associated with poor response rate to immunosuppressive therapy indicates a more basic disorder of cell cycle regulation including increased rates of apoptosis or a greatly reduced stem cell pool. The initial difference in the proliferation rate as evident by SPF between patients with good treatment response and those with poor response may provide additional prognostic information and equally important, shed light on the pathogenetic heterogeneity among patients with AA.

In conclusion, SPF at the time of diagnosis may provide, independently of the peripheral blood count, information on the expected response to immunosuppressive therapy as well as propensity to evolve into MDS/AML. The assessment of the proliferative activity by measuring SPF may add valuable information on the clinical course and may contribute to therapeutic decisions.

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