Interphase FISH Analysis of Bone Marrow Cells in Aplastic Anemia Patients Following Immunosupression Therapy

Aplastic anemia is a disease that severely impairs hematopoiesis, the bone marrow's production of blood cells, and results in pancytopenia, an extreme reduction in the concentration of red and white blood cells and platelets.[1] Immunosuppressive therapy with medications such as antithymocyte globulin and cyclosporine are used to treat these patients. Previous studies suggest that the presence of specific chromosomal abnormalities, trisomy 8 and monosomy 7 in the bone marrow of treated patients are correlated with different outcomes from therapy.[2]

Studies are ongoing that address the correlation of these specific aneuploides with patient outcome. In this study, fluorescent *in situ* hybridization (FISH) was used to assess the copy numbers of chromosomes seven and eight in bone marrow samples from patients treated with immunosuppressive therapies. We are interested in improving the sample preparation part of the bone marrow FISH method protocol so that results will be easier to score and less bone marrow sample volume will be necessary. As a part of this summer study, we will develop a new method for these lab studies using a cytocentrifuge to concentrate the bone marrow samples on each slide to a central location. The overall purpose of this study is to further understand the effects of immunosuppressive therapies on aplastic anemia patients.

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Introduction

Aplastic anemia may directly result from the destruction of stem cells caused by radiation, cytotoxic chemotherapy and chloramphenicol and benzene exposure.[1] Less than 10% of cases occur after a person acquires seronegative hepatitis.[3] Half of all cases, however, are idiopathic and have no apparent cause.[4] Once a person acquires aplastic anemia, his or her ability for hematopoiesis is greatly reduced, ultimately resulting in pancytopenia. One major complication of aplastic anemia is its evolution to clonal hematological diseases such as myelodysplasia. Myelodysplastic syndromes are clonal stem cell disorders that often involve karyotipic abnormalities concerning chromosomes five, seven, or eight.[5] Monosomy 7 and trisomy 8 appear to develop over time in those patients treated with immunosuppressive therapies.[2] The purpose of this study was to perform fluorescence *in situ* hybridization (FISH) testing on bone marrow samples to determine if samples with aneuploidies were related to patient outcome which may ultimately help to understand the effects of immunosuppressive therapies on patients with aplastic anemia.

Methods

Bone marrow samples from 13 patients for this study were obtained from the laboratory of Dr. Elaine Sloand, NHLBI, NIH. The diagnosis of aplastic anemia was established by bone marrow biopsy and peripheral blood counts according to the International Study of Aplastic Anemia and Agranulocytosis criteria. The patients with a normal chromosome number at diagnosis were treated with immunosuppressive therapy using antithymocyte globulin and cyclosporine with or without granulocyte colony-stimulating factor.

In order to collect pellet from the samples, we first centrifuged them at 900 rpm for 10 minutes and then removed the supernatant. A fresh fixative composed of a 3:1 glacial acetic acid/methanol was added and allowed to incubate for 20 minutes. The samples were then centrifuged and the pellet was collected and resuspended in 1 mL of fixative and allowed to incubate for 20 minutes. After being centrifuged a third time, the pellet was resuspended twice in a phosphate buffered saline (PBS) solution for 10 minutes. Next, the samples were centrifuged and the resulting pellet was resuspended in a final aliquot of 1 mL of PBS. A cytocentrifuge (Shandon Instruments) was used to apply the sample to the slides. For each slide, 400 μL of PBS was loaded into each chamber, followed by 200 μL of sample. It was then spun at 1500 rpm for five minutes. The cytocentrifuge allowed us to concentrate the sample to a central location to make scoring the slides easier.

FISH testing was performed using centromeric probes Spectrum Green™ and Spectrum Orange™ to label chromosomes seven and eight (Vysis Inc, Downers Grove, IL). The slides were first rinsed in 2× SSC in a water bath of 36°C for 30 minutes. Afterward, the slides were washed for two minutes in each of a series of three ethanol solutions of concentrations of 70%, 80%, and 95%. After they had completely dried, 100 mL of a denaturation solution composed of a 30 μL of 20×

SSC and 70 μL of formamide was applied to each slide, followed by a cover slip. The slides were then denatured on a Hybrite (Abbott Labs, Napreville, IL) at 76°C for two minutes. Next, the cover slips were removed and the slides were dehydrated in a graded ethanol series as above. At the end of this ethanol series the slides were allowed to dry completely, and four μL of probe solution with a 1:9 ratio of probe to hybridization buffer was added to each slide. Cover slips were then added and glued on. The slides were incubated at 37°C for an hour and then washed in a 0.2× SSC solution with Tween 20 for five minutes at 70°C after their coverslips were removed. The slides were then washed in a final 4× SSC 0.05% Tween 20 for five minutes at room temperature. After the slides dried, DAPI was added along with a final coverslip.

An Olympus fluorescence microscope equipped with an Applied Imaging Cytovision imaging system was used to visualize the signals on the slides. Each slide was read by three people.

Discussion

The cytocentrifuge was useful when used to create slides of lower density samples because it allowed us to concentrate the cells within a specific area, making them easier to read. Some samples, however, were so dense that using the cytocentrifuge hindered our ability to read the slides produced because the cells congregated into an unidentifiable mass. Ultimately, the cytocentrifuge sped up the counts. Others who wish to use cytocentrifuge techniques may want to consider not using it for high-density samples.

Cohybridization of the probes was very efficient because it allowed us to count the copies of chromosome seven and chromosome eight within the same cells. This allowed us to see the frequency of different chromosomal abnormalities together and separately.

Further studies will likely include more analysis and perhaps some studies on the genes located on chromosones seven or eight that confer some advantages to the patient.

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