Chapter 82

Aplastic Anemia, Apoptosis and Telomeres: Insight into Missing Link and Clinical Implications

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INTRODUCTION

Aplastic Anemia (AA) is a life-threatening disorder characterized by peripheral blood pancytopenia in combination with a hypocellular bone marrow. The disorder arises due to specific failure of bone marrow precursor cells or pluripotent stem cells to produce adequate amount of mature hematopoietic cells. It may arise as single cell cytopenias showing failure of one of the committed cell line and later on, can lead to marrow aplasia. The hematopoietic cells are replaced by fat cells.

About 75% of cases of AA are classified as idiopathic and the main cause remains unexplained. In about 15% of cases, a drug or infection can be identified that precipitates the event, although why only some individuals are susceptible is unclear. In about 5–10% of cases, the disease is familial (constitutional).

Initially, researchers emphasized putative etiologies and the relationship of AA to chemicals like benzene and idiosyncratically, to drug exposures, most notably chloramphenicol has been observed. In last few decades, the understanding of AA has centered around a unified immune mechanism of hematopoietic cell destruction, based on the observations that blood counts improved in majority of patients who were treated with immunosuppressive therapies. Most recently, acquired form of AA has been linked to inherited genetic mutations and the molecular biology of the disease has been elucidated based on dysfunction of the affected genes.

EPIDEMIOLOGY

The incidence of AA in the West is two per million and is about two-to threefold higher in Asia. Recently completed Thai National Heart, Lung, and Blood Institute Aplastic Anemia Study performed in Bangkok has shown that benzene and pesticides, while significantly associated, accounted for only a small number of cases and medical drugs have a negligible role in Asia. From India, scattered data is available.

Aplastic anemia has been identified as a disease of the young. However, the disease demonstrates two peaks, with most patients presenting between 15 years and 25 years or after age 60 years. In children and young adults, acquired AA should be distinguished from the inherited forms of bone marrow failure such as Fanconi’s anemia and Dyskeratosis congenita.

PATHOPHYSIOLOGY

In most proportion of cases, AA behaves as an immune-mediated disease. Detailed pathways have been elucidated involving the effector T lymphocytes and the target hematopoietic stem and progenitor cells.

The responsiveness of AA to immunosuppression remains the best evidence for an underlying immune pathophysiology. Majority of patients of AA show hematologic response after T-cell depletion with antithymocyte globulins (ATG). However, not all patients of AA show response to immunosuppressive therapies. Failure to respond to immunosuppression has been interpreted as either due to an alternative pathophysiologic mechanism operating in these nonresponder patients or due to immunological mechanisms that are not susceptible to current immunosuppressive therapies.

It has been well-documented in experimental studies that removal of lymphocytes from AA marrows improved colony numbers in tissue culture and their addition to normal marrow inhibited hematopoiesis in vitro. These effector cells are activated cytotoxic T-cells expressing Th 1 cytokines especially gamma interferon. Why T-cells are activated in AA remains unclear. HLA-DR2 is overexpressed among patients, thereby suggesting a role for antigen recognition. Polymorphisms in cytokine genes associated with increased immune response are also more prevalent.

It has been seen that white blood cells in AA have short telomeres. However, discovery that X-linked form of dyskeratosis congenita was due to mutations in DKC1, indicated a genetic basis for telomere deficiency. Some patients with apparently acquired AA have mutations in telomerase ribonucleic acid component (TERC) and telomerase reverse transcriptase (TERT). Now it is well-documented that inherited mutations in genes that repair or protect telomeres are more prevalent.

It has been seen that telomere shortening was the result of a “stressed” hematopoietic stem cell, which overproliferates in response to marrow destruction. The recent recognition of genetic defects in telomeres and telomere repair in AA has implications for therapy, prognosis, monitoring and genetic counseling. Patients of AA with short telomeres are mainly those who do not respond to immunosuppression. Measurement of telomere length of peripheral blood leukocytes is a simple screening clinical assay. However, gene penetration, onset of symptoms and signs either early or late in life, disease anticipation, the ultimate severity of clinical manifestations and the organs affected are influenced by incompletely defined added environmental, epigenetic or genetic modifiers.
Telomere Biology

In humans, telomeres are hexameric repetitive DNA sequences (TTAGGG in the leading strand and CCCTAA in the lagging strand) capped by specific proteins at the extremities of linear chromosomes (Figures 1A to C). The leading strand (G-rich) ends in a single-stranded 3’ overhang, which is generated by processing of the lagging (C-rich) strand. The single-stranded 3’ overhang folds back into the telomeric DNA, invades the double-helix and anneals with C-rich strand, forming a T-loop, which hides the ends of chromosomes. Telomeres are coated by a group of six proteins called shelterin. Shelterin serves as a signal that allows the cellular DNA-repair machinery to differentiate telomeres from DNA double-stranded breaks.

Telomeres cannot be fully duplicated during cell division. As a result of this insufficiency called the “end-replication problem”, there is shortening of the telomere after every cell division (Figure 2). Additionally, telomeres are further shortened by postreplicative DNA processing or by direct damage caused by reactive oxygen species. When critically short, telomeres signal cell proliferation arrest, senescence and apoptosis. When the cell bypasses these inhibitory pathways and continues to proliferate, extremely short telomeres lose their function of chromosomal protection. Either way, the cell undergoes programmed death.

Telomerase

To prevent telomere shortening, highly dividing cells such as hematopoietic stem cells express the enzyme telomerase. Telomerase is a specialized reverse transcriptase that uses a specific ribonucleic acid (RNA) molecule, TERC as the template to extend the 3’ end of the leading strand by adding TTAGGG repeats. The telomerase complex is composed of TERT (reverse transcriptase...
Hematology

The end replication problem:

Telomeres shorten with each S phase

DNA replication is bidirectional polymerases move 5' to 3' requires a labile primer

Each round of DNA replication leaves 50–200 bp DNA unreplicated at the 3' end

Figure 2: The end replication problem

enzyme), TERC and the ribonucleoprotein dyskerin (encoded by DKC1 gene), which is important for the RNA component folding and stability. NOP10, NHP2 and GAR are other proteins that are associated with the telomerase complex. These proteins stabilize the TERC molecule and play a role in ribosome biogenesis and messenger RNA processing. Most mature cells do not express telomerase and hence cannot elongate their telomeres. Stem and progenitor cells contain very low levels of telomerase but telomerase expression is tightly regulated.

Telomere Shortening and Aplastic Anemia: Clinical Implication

Most cases of acquired AA result from immune-mediated destruction of hematopoietic stem cells. However, a minority of patients has heterozygous mutations in genes encoding the telomerase components TERT or TERC (5–10%). These patients of AA with telomerase mutations do not respond adequately to immunosuppressive therapy. The majority of these AA patients respond, at least transiently, to androgen therapy since androgens stimulate telomerase expression in hematopoietic cells by their aromatization into estrogens. The telomere length is critical in therapy decision making. Therefore, it is essential to recognize early, those patients of AA with telomerase mutations, so that appropriate therapy (stem cell transplantation) may be offered to them, since immunosuppressive therapy (ATG) would not result in disease reversal in these patients.

Maintenance of adequate telomere length is crucial for hematopoiesis, and excessive telomere erosion as a result of mutations in telomerase underlies the pathogenesis of nearly one-third of patients with acquired AA. Identification of such patients with telomerase mutations is crucial in treatment decision-making (Figure 3).

PILOT STUDY OF TELOMERASE MUTATIONS IN APLASTIC ANEMIA

From the foregoing discussion, it is clear that mutations in TERT and short telomeres are pathophysiologic mechanisms in some of the patients of AA. No study has been done previously to identify mutations in TERT region in AA patients in Indian subcontinent population. We have performed mutational analysis study in five patients and six controls.

METHODOLOGY AND EXPERIMENTAL DESIGN

Objective of the Present Study

Detection of telomerase mutations in patients of AA attending SMS Hospital.

Blood Sample Collection

Five milliliter of peripheral blood samples (venous blood) were obtained from confirmed patients of apparently acquired AA attending SMS Hospital and who fulfilled the bone marrow and blood count criteria of the International Agranulocytosis and Aplastic Anemia Study. Patients or their attendants provided written informed consent for genetic testing according to protocol approved by the Institutional Ethics Committee.

Criteria Design

Patients were selected from Hematology OPD clinic in SMS Hospital and who had fulfilled the blood count and bone marrow criteria of the International Agranulocytosis and Aplastic Anemia Study.

Number of subjects: 11 [six controls (C1 to C6) and five patients (P1 to P5)]

Inclusion criteria—Age: 10–70 years

Blood and bone marrow picture should have:

- Granulocytes less than 0.5 × 10⁹/L
- Platelets less than 2.0 × 10⁹/L
- Reticulocytes less than 1% (corrected)
- Marrow cellularity less than 25%.

DEOXYRIBONUCLEIC ACID EXTRACTION

Genomic DNA extraction was done from whole blood of controls and patients using “Qiagen Genomic DNA Isolation Kit.” Lysis of blood sample was done by incubation in a lysis solution and pure genomic DNA was eluted under high salt concentrations. Quality of purified DNA was assessed by Agarose Gel Electrophoresis. Purified DNA sample was collected and stored at 4°C.

Polymerase-Chain-Reaction Amplification

Polymerase-chain-reaction (PCR) amplification of genes encoding the telomerase complex—namely DKC1, NOP10, NHP2 and TERT was performed with DNA samples extracted from peripheral blood. Primer sequences and PCR conditions are given in supplementary appendix. Three types of PCRs were done:

1. Conventional PCR
2. Gradient PCR
3. Touchdown PCR.

All sequences were determined in both directions and mutations were identified. Approximate 22 sets of primers were used for amplification of TERT, DKC1, NOP10 and NHP2 genes fragments. After amplification, PCR products were analyzed by using Agarose gel electrophoresis system (Bio-Rad) for the comparison of our amplicon and the length of 500 bp DNA ladder (supplementary appendix). Polymerase-chain-reaction products were purified with a QIA quick PCR purification kit (Qiagen).

Deoxyribonucleic Acid Sequencing and Mutational Analysis

Polymerase-chain-reaction primers were used for DNA sequencing, and sequencing products were analyzed in an automated genetic-sequence analyzer (ABI Prism 3100, Applied Biosystems) at Xcelris Labs Limited, Ahmedabad.

Mutations in the probands and controls were confirmed by bidirectional sequencing. Sequences were inspected with the use of CLC main workbench version 5.1 and variants were identified with control sequence available at public databases on National Center for Biotechnology Information and Ensemble.

Bioinformatics Analysis

Basic Local Alignment Search Tool was used for alignment of our query sequence against sequences in a molecular database.
Clustal X is a version of Clustal W with a graphical user interface. This program was used for multiple sequence alignment.

Statistical Analysis
Statistical methods were applied to judge the significance of matches. Differences in the frequencies of coding-sequence variations between samples from patients with AA and those from controls were evaluated by means of z-test and chi-square tests.

RESULTS

Controls and Aplastic Anemia Patients
Deoxyribonucleic Acid Concentrations
All five patients with AA had low DNA concentration as compared to control (Table 1).

Mutations in Noncoding Regions
Out of the five patients, patient P1 carried two mutations in noncoding regions (P1: CTG instead of CAG and TTG instead of TCG with primer sets F2/R2). Among the remaining four patients, one mutation was found in patient P3 (P3: ACC instead of AGC with primer sets F5/R5). Only one control C2 had mutation in noncoding region (CCC instead of CGT, ACA instead of AGA and CCT instead of CGT) with primer F7. Multiple sequence alignment (MSA) of some selected regions is listed in Table 4 of the supplementary appendix. The mutations identified from sequencing have been listed in Table 2.

Mutations in Coding Regions
Most mutations were observed in exon 12 in TERT gene. Substitution mutations were found in four patients P1 (T > G), P2 (A > G), P3 (A > G) and P5 (A > G) for the same codon-901 with primer sets F8/R8. We also found new mutation in controls C1 (A > G) and C3 (T > G). Other important substitution mutation was found in DKC1 gene in exon 15 codon-S82 in C1 (A > G) with primer sets of F18/R18.

Table 1: Information of deoxyribonucleic acid samples

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Sample name</th>
<th>260/280 ratio</th>
<th>DNA* Concentration (ng/µL)</th>
</tr>
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<tbody>
<tr>
<td>1.</td>
<td>C1</td>
<td>1.78</td>
<td>91</td>
</tr>
<tr>
<td>2.</td>
<td>C2</td>
<td>1.91</td>
<td>93</td>
</tr>
<tr>
<td>3.</td>
<td>C3</td>
<td>1.89</td>
<td>89</td>
</tr>
<tr>
<td>4.</td>
<td>C4</td>
<td>1.77</td>
<td>98</td>
</tr>
<tr>
<td>5.</td>
<td>C5</td>
<td>1.83</td>
<td>96</td>
</tr>
<tr>
<td>6.</td>
<td>C6</td>
<td>1.74</td>
<td>119</td>
</tr>
<tr>
<td>7.</td>
<td>P1</td>
<td>1.77</td>
<td>47</td>
</tr>
<tr>
<td>8.</td>
<td>P2</td>
<td>1.73</td>
<td>42</td>
</tr>
<tr>
<td>9.</td>
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<td>1.76</td>
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</tr>
<tr>
<td>10.</td>
<td>P4</td>
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</tr>
<tr>
<td>11.</td>
<td>P5</td>
<td>1.74</td>
<td>57</td>
</tr>
</tbody>
</table>

*DNA: Protein concentration ratio is not varying (controls and patients also have ideal value 1.7–1.9) but DNA concentration is varying too much.

Figure 3: Consequences of telomere erosion in the cell. Telomeres inexorably shorten with every cell division, and telomere attrition is an inevitable physiological consequence of aging. Telomere shortening also may be iatrogenic; for example, telomere shortening occurs after bone marrow transplantation, in which highly proliferative hematopoietic stem cells and progenitor cells reconstitute hematopoiesis. Environmental factors also may accelerate telomere loss. In addition, telomere attrition may be genetic; there may be an inherited inability to elongate telomeres as a result of mutations in components of the telomerase complex. When telomeres become critically short, inappropriately capped chromosomes or telomere-free ends emerge, which lead to cell senescence or apoptosis. If the cell overrides senescence and continues to proliferate (e.g. because of inactive p53), uncapped telomeres may cause end-to-end fusion of chromosomes, breakage-fusion-bridge cycles, aneuploidy, and chromosomal translocations (Source: Adapted from NEJM. 2009;361:2353-65.)
Patients P1 (Ser/Ile) and P2 (Ser/Lys) had novel nonsynonymous mutations (i.e. mutations that introduce an amino acid change in the corresponding protein) in TERT. The remaining two patients P3 (Ser/Asn) and P5 (Ser/Asn) had similar but nonsynonymous mutations in TERT. No mutations were found in NOP10 and NHP2 genes analyzed.

Statistical Analysis
Statistical methods were applied to judge the significance of matches. Differences in the frequencies of coding-sequence variations between samples from patients with AA and those from controls were evaluated by means of z-test and chi-square test. There was no significant difference observed (Z = 0.666; P = 0.506) between two groups (controls and patients) with respect to number of study participants having mutations.

DISCUSSION
In this study, nonsynonymous mutations observed in the TERT genes are in concordance with several earlier reports from the western world.

In one of the previous study on AA, 3 of 200 (1.5%) patients were found to have mutations in human telomerase RNA component (Yamaguchi et al. 2003) and in an additional study on AA approximately 4% (7/200) of patients with apparently acquired AA had heterozygous TERT nonsynonymous mutations that disrupted telomerase activity by haplinsufficiency, causing short telomeres of leukocytes and a hematopoietic stem cell compartment of limited proliferative capacity (Yamaguchi et al. 2005).

In this study, we found mutation in exon 12 in TERT gene located within the reverse transcriptase domain. There are different types of mutations present in different subjects in the same codon 901. P1 and P2 have same mutation in codon 901 where serine is replaced with isoleucine and lysine respectively in the C-terminal region of TERT (Ser/Ile and Ser/Lys). Patients P3 and P5 have another similar mutation (Ser/Asn), in the C-terminal of TERT. These are all substitution mutations (i.e. substitution of amino acid).

Other important substitution mutation was found in exon 15 in DKC1 gene. Codon-582 had replacement (A > G) in control C1. None of the two controls (C1 and C3) who had mutations showed any hematological abnormalities and C1 who had mutations in both TERT and DKC1 genes showed none of the physical signs of dyskeratosis congenita.

It is hypothesized that mutations in genes of the telomere repair complex reduce the size of the hematopoietic stem cell compartment and the regenerative capacity of the marrow, making carriers of gene mutations susceptible to the development of marrow failure and affecting the course of AA once it develops.

Clinical Response to Immunosuppressive Treatment
Of the five patients of AA on cyclosporine therapy, three have succumbed to complications of AA (bleeding and sepsis) and another is heavily transfusion-dependent. Patient P4 who did not have any mutations is doing fairly well with cyclosporine and does not require transfusion support at present.

CONCLUSION
The present study was undertaken to evaluate the mutation spectrum in the genes implicated in AA, i.e. TERT, DKC1, NOP10 and NHP2 on small case-control group (5 + 6). We have been successful in finding mutations in TERT, TERC and DKC1 while no population-specific mutations were found in NOP10 and NHP2. The statistical significance of these mutations is difficult to establish.
as the sample size was too low. However, none of the patients with TERT mutations had a response to immunosuppressive therapy. We have validated our approach for undertaking a larger study.

**FUTURE ASPECTS**
While the connection between telomere shortening, telomere dysfunction and AA is now abundantly clear, this knowledge has not yet translated to disease management. It is possible that specific mutations in one or more genes could influence the choice of therapy: immunosuppressive treatment or stem cell transplantation. For this reason, measurement of telomere length and testing for mutations in telomerase genes could be useful in the management of acquired AA.

**REFERENCES**