行政院國家科學委員會專題研究計劃成果報告

計畫名稱：再生不良性貧血與驟發性夜間血色素尿症之GPI-ANCHORED蛋白與PIG-A基因序列變化之研究

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註：整合型計畫總報告與子計畫成果報告請分開編列各成一冊，彙整一起繳送國科會。

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中文摘要:

驟發性夜間血色素尿症是一種後天性溶血性貧血，主要是造血幹細胞上GPI anchor合成缺失所致。位於X-染色體上的*PIG-A*基因參與了GPI anchor合成中的第一個步驟。驟發性夜間血色素尿症被認為與此基因發生突變有相當大的關連性。到目前為止的研究報告顯示所有被研究的此種病患之*PIG-A*基因都有突變發生。我們曾整理並分析了四個罹患驟發性夜間血色素尿症的台灣人之臨床表現過程及*PIG-A*基因。我們鑑定出五個之前沒有被報告過的 *PIG-A*基因變異，包括一個核甘酸的置換，如：-342(C→G), codon 335(GGT→AGT) 及codon 405(GCT→GTT)，和核甘酸讀架改變，如：codon 22(GGA→G-A) 及codon 356(TGT→TGTT)。其中-342 (C→G)被認定是基因多樣性與疾病無關。這四位病患當中的三位先前都有再生不良性貧血症的病史.本實驗的目的，乃藉分析正常人和上述幹細胞疾病病人之周邊血球表面的GPI-anchored蛋白表現情形，並加以比較，以探討GPI anchor在這些病人身上所扮演的角色。我們分別將所需的各種血球細胞分離、稀釋，加入單株抗體反應(紅血球：CD59，顆粒球：CD13及CD67，T型淋巴球：CD2及CD59，B型淋巴球：CD19及CD48)，洗淨後，利用流式細胞計數儀做GPI-anchored蛋白的分析. 結果發現PNH病人周邊血液之紅血球、顆粒球及B型淋巴球皆出現明顯的GPI-anchor蛋白缺乏；而少數正常人以及部分MDS病人有小部分CD67表現稍弱的顆粒球，其餘細胞的GPI-anchored蛋白則表現正常.同時也發現，少數正常人有一群GPI-anchored蛋白表現較弱的細胞，由此推論可能在少部分正常人體內存在著一些GPI-anchored蛋白表現較弱的細胞。另外，3位MDS病人的顆粒球也出現GPI-anchor 蛋白缺乏.

英文摘要:

Paroxysmal nocturnal haemoglobinuria (PNH) is an acquired haemolytic disorder caused by deficient biosynthesis of the glycosyl phosphatidylinositol (GPI) anchor in haemopoietic stem cells. *PIG-A*, an X-linked gene that participates in the first step of GPI-anchor synthesis, is responsible for PNH. Various abnormalities of the *PIG-A* gene have been demonstrated in all patients with PNH so far examined. In past study, we characterized the somatic mutations in *PIG-A* gene in four Taiwanese patients with PNH. We identified five novel mutations in the *PIG-A* gene, three single nucleotide substitution mutations (-342, C→G, codon 335, GGT→AGT and codon 405, GCT→GTT) and two frameshift mutations (codon 22, GGA→G-A and codon 356, TGT→TGTT) in the *PIG-A* gene. The -342 mutation was judged to be a polymorphism. Furthermore, three patients had previous clinicopathologic evidence in which suggested aplastic anaemia (AA), before the development of PNH. We suggest that the somatic *PIG-A* gene mutations highlight a subgroup of AA having a pathogenetic link with PNH. The puropse of this study is to analyze and

compare the presence of GPI-anchored proteins in normal adults and patients with AA and MDS. RBC, granulocytes, and lymphocytes were isolated and analyzed the GPI-anchored proteins on the cell membrane by flowcytometer. Our results showed that RBC, granulocyte, and B-lymphocyte expressed deficient GPI-anchored proteins in PNH patients. A small number of normal adults and patients with MDS had a few abnormal granulocytes.

Introduction

Paroxysmal nocturnal haemoglobinuria (PNH) is an acquired clonal haemolytic anaemia with protean clinical manifestations, including intravascular haemolysis, intermittent haemoglobinuria, venous thrombosis, infections, and a tendency toward bone marrow aplasia (Rotoli and Luzzato, 1989). The intravascular haemolysis is caused by an increased susceptibility of red cells to complement-mediated damage to the plasma membrane. This unusual susceptibility to complement is due to an absence of molecules that regulate the complement cascade acting on the plasma membrane. These molecules include (1) DAF (decay accelerating factor, CD55) which regulates the formation of the "convertase" complex of C3bBb and C4b2a, and (2) MIRL (membrane inhibitor of reactive lysis, CD59) which controls the formation of the membrane-attacking complex C5b-9 (Rotoli et al, 1993).

Membrane-bound proteins may be anchored to the plasma membrane by a transmembrane segment of amino acids reacting with lipid of the bilayer, or via a glycosylphosphatidylinositol (GPI) anchor. The latter are called GPI-anchored proteins (Doering et al, 1990). It has been noticed that, in addition to CD55 and CD59, many other GPI-anchored proteins, such as CD14, CD16, CD24, CD48, CD58, CD67, CD73, and acetylcholinesterase, are deficient in various haematopoietic cells of PNH patients (Rosse, 1990). These findings have led to the conclusion that insufficient biosynthesis of the GPI anchor is the biochemical basis of PNH (Hirose et al, 1992; Armstrong et al, 1992).

PNH often manifests as a late clonal complication in patients with aplastic anaemia (AA) (Rotoli and Luzzatto, 1989). Whether AA and PNH are different forms of the same disease is as yet unresolved. However, GPI-anchor deficiency was identified at a high frequency (52%) in acquired AA patients, in at least one cell lineage (Schrezenmeier et al, 1995). Some AA patients have been identified as having GPI-anchored protein deficiency, caused by somatic mutation of *PIG-A* gene, before clinical manifestations of PNH developed (Nakakuma et al, 1995; Griscelli-Bennaceur et al, 1994).

Materials and Methods

Patients:

In this study, 30 normal controls and 18 patients with AA, MDS and PNH were examined. GPI-anchored proteins analysis of fresh blood was carried out.

Monoclonal antibodies and GPI-anchored proteins analysis with flow cytometry:

Mouse anti-human CD2-RPE, CD13-RPE, CD19-RPE, CD14-FITC, CD48-FITC, CD55-FITC, CD59-FITC, CD67-FITC as well as mouse anti-human IgG1-FITC, IgG2a-FITC, IgG1-RPE, IgG2a-RPE and IgG2b-RPE will be purchased from Serotec Ltd, Oxford, England.

For RBCs, we use CD55 and CD59; for monocytes, we use CD13 and CD14 (GPI-anchored protein); for granulocytes, we use CD13 and CD67 (GPI-anchored protein); for T-lymphocytes, we use CD2 and CD59 (GPI-anchored protein); for B-lymphocytes, we use CD19 and CD48 (GPI-anchored protein).

Results and Discussion:

Table 1 showed various GPI-anchored proteins expressed on blood cells in normal controls and patients with PNH, AA and MDS. From these data, we found that there were a few GPI-anchor deficiencies on some blood cells in normal controls and patients with AA and MDS, in addition to patients with PNH.

The clinical response to antilymphocyte globulin (ALG) and cyclosporine A (CsA) appears to be very good in most AA patients (Schrezenmeier et al, 1992). In contrast, this treatment exacerates symptoms in AA patients with GPI-anchor deficiency (Schubert et al, 1994). It was also noted that this treatment could shorten the interval between the AA phase and the phase with a positive Ham test (3-18 months; Nagarajan et al, 1995). Three of these patients we have studied were diagnosed as having AA previous to the diagnosis of PNH, and one of them was associated with thrombocytopenia in a later stage. Futhermore, these patients had not been treated by such strong immunosuppresants and they had long disease-free intervals (1-5 years) between the AA phase and the PNH phase.

The mechanism of AA-PNH phase switching is still unknown. According to the theoretical possibility that low numbers of PNH clones may be present in normal subjects (Dacie, 1980), we suggest a "two-stage hypothesis" as follows. The first, after predisposing factors attack, all bone marrow cells may be affected and AA is thus established; at this time, the PNH clones lacking CD58 (LFA-3) (Dustin and Springer, 1991), a GPI-anchored protein, might escape from the immune attacks and the following growth inhibitory activity (Bessler et al, 1994b). The PNH clones would thus be relatively enhanced in number. The Second, the immuno-suppresant therapy with ALG and CsA might provide a microenvironment

which favors the growth of the PNH clones compared with normal clones in bone marrow and thereby expedites the progression of disease. The increased fraction of PNH clones would result in variable PNH expression depending on the proportion of these cells in various blood elements.

Nevertheless, it remains unclear how the haematopoietic PNH clone can expand and become dominant, that is, does this clone have an intrinsic growth advantage like the leukemic clone, or an extrinsic survival (or selective) advantage like the thalassemic gene?

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Table 1. Various GPI-anchored proteins expressed on blood cells in normal controls and patients with PNH, AA and MDS.

	RBC CD59 (-) %	Gran CD67 (-) %	T cell CD59 (-) %	B cell CD48 (-) %
Normal	0	0.32±0.5	0.52±0.58	0.94±1.02
MDS	0	2.67±3.47	0.08±0.2	0.47±0.31
AA	0	0.22±0.28	0.77±1.26	0.75±0.86
PNH	69.62±3.22	83.62±29.37	5.44±6.61	20.1±10.56