Molecular analysis of the GPI-deficient clonal hematopoiesis in paroxysmal nocturnal hemoglobinuria.

von dem Fachbereich Chemie
der Universität Hannover
zur Erlangung des Grades
Doktor der Naturwissenschaften
Dr. rer. nat.

genehmigte Dissertation
von
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2002

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Tag der Promotion:

5. Februar 2002

To my parents

Diese Arbeit wurde in der Zeit von Juli 1999 bis Dezember 2001 in der Arbeitsgruppe von PD Dr. J. Schubert, Abteilung Klinische Immunologie, Medizinische Hochschule Hannover (Leiter: Prof. Dr. R.E. Schmidt) mit Unterschtützung der DFG, Projekt Schu 713/7-1 durchgeführt.

Zusammenfassung

Die paroxysmale nächtlicher Hämoglobinurie (PNH) ist ein erworbener klonale Defekt von hämatopoietischen Stammzellen charakterisiert durch die Defizienz Glycosylphosphatidylinositol (GPI)-verankerten Oberflächen-proteine auf den peripheren Blutzellen. Diese Proteine schließen komplementregulatorischen Molekülen CD55 und CD59 ein, und das Fehlen an ihnen erklärt die Empfindlichkeit der Erythrozyten vom PNH Patienten gegenüber der Komplement-vermittelten Lyse, wodurch eine intravasale Hämolyse bewirkt wird. Dieser Defekt wurde Mutationen innerhalb des *PIG-A* Gens zugeschrieben, das für ein in GPI-Anker-Biosynthese beteiligtes Protein kodiert.

Jedoch ist der Mechanismus, durch den die GPI-defizienten Stammzellen innerhalb des Knochenmarks expandieren noch nicht geklärt worden. Mutationen in *PIG-A* Gen sind für die Entwicklung der PNH nicht ausreichend, da sie durch der GPI-Defizienz allein nicht expandieren. Deshalb sind zusätzliche Veränderungen wie Alterationen wahrscheinlich, die zur klonalen Expansion von GPI-defizienten Knochenmark Stammzellen bei der PNH führen.

Um die molekulare Grundlage der klonalen Expansion bei der PNH zu analysieren, identifizierten wir mehrere differentiell exprimierte Gene im Vergleich normaler und GPI-defizienter Zellen von PNH Patienten durch Anwendung zweier divergierender Ansätze: RNA Fingerprinting und cDNA array-Hybridisierung. Aus eine Serie von 26 differentiell exprimierten Genen haben wir 2 Kandidaten (EGR-1 und TAXREB107) für weitere Untersuchung ausgewählt, da sie eine ausgeprägtere Alteration des Expressionsniveaus aufweisen. Außerdem wurde ihnen bereits eine Rolle in Regulierung der Hämatopoese zugeschrieben. EGR-1 ist hochreguliert in Granulozyten aller bis jetzt analysierten PNH Patienten. Im Gegensatz dazu wurde eine signifikante Hochregulation von TAXREB107 nur in einigen unserer PNH

Patienten beobachtet, die die PNH im Verlauf einer aplastischen Anämie entwickelt haben. In weiteren Analysen konnten wir ein mögliches sekundäres Ereignis als Ursache der beobachteten Überexpression ausschließen. Außerdem ist ein ähnliches Expressionsniveau in Fällen anderer klonaler Erkrankungen wie MPS und MDS identifiziert worden. Um den Hintergrund der entdeckten Änderung des Expressions Niveaus zu analysieren, haben wir die chromosomale und molekulare Organisation des EGR-1 Gens und seiner Transkripte untersucht. Wir entdeckten keine Genamplifikation und keine Änderungen der primären Struktur im Bereich der Promoterregion. Jedoch offenbarten sich einige Mutationen der Kodierenden Region des EGR-1 Gens im Bereich der Zink-Finger Domänen. Die indentifizierten Mutationen im sensiblen Funktionsbereich könnten zur Hochregulation der Gen Expression beitragen oder sogar für sie verantwortlich sein.

Zusammengefaßt zeigen unsere Daten, daß zusätzliche genetische Änderungen neben Mutationen in *PIG-A* bei den GPI-defiziente Zellen von PNH-Patienten vorhanden sind. Außerdem könnten solche genetischen Änderungen in Zellaktivierungs-Repertoire zur klonalen Expansion von GPI-defizienten Zellen bei der PNH beitragen.

Paroxysmale nächtliche Hämoglobinurie; EGR-1; TAXREB107

Summary

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired clonal defect of hematopoietic stem cells characterized by appearance of peripheral blood cells deficient in glycosylphosphatidylinositol (GPI)-anchored surface proteins. These proteins include complement inhibitors CD55 and CD59, and lack of them explains the hypersensitivity to complement-mediated lysis of red cells in PNH patients, resulting in intravascular hemolysis. This defect could be attributed to mutations within the *PIG-A* gene encoding for a protein involved in GPI-anchor biosynthesis.

However, the mechanism enabling the GPI-deficient stem cell in PNH to expand within the bone marrow and contribute considerably to the hematopoiesis has not been clarified yet, because PIG-A gene mutations are not sufficient for the development of PNH, and GPI-deficient cells do not expand by themselves. Therefore, a second event is very likely, such as additional genetic alterations, leading to clonal expansion of GPI-deficient bone marrow stem cells in PNH.

In order to elucidate the molecular basis of clonal expansion in PNH we identified several genes differentially expressed in normal and GPI-deficient cells of PNH patients by combination of two divergent approaches: RNA fingerprinting and cDNA array hybridization. From a set of 26 differentially expressed genes, we have chosen 2 candidates (EGR-1 and TAXREB107) for further investigation according to their more pronounced alteration of expression level and their previously described role in regulation of hematopoiesis. EGR-1 is upregulated in granulocytes of all PNH patients analyzed so far. In contrast, significant upregulation of TAXREB107 is present only in some of our PNH patients, who developed PNH after aplastic anemia. Further analysis confirmed overexpression of these genes in PNH and excluded a possible secondary event character of the observed overexpression. Moreover, similar level of expression in cases of other clonal

diseases such as MPS and MDS has been identified. In order to analyze detected change in expression level, we studied chromosomal and molecular organization of EGR-1 gene and transcript. We detected no gene amplification, no changes in primary structure of promoter area. However, we revealed some mutations in the coding region of *EGR-1* gene corresponding to the area encoding for zinc-finger domains. It might be that these identified molecular alterations can contribute to or even be responsible for the upregulation of *EGR-1* gene expression.

Taken together, our data indicate that additional genetic alterations apart from *PIG-A* mutations are present in PNH. In addition, such genetic changes in cellular activating repertoire could contribute to clonal expansion of GPI-deficient cells in PNH.

Paroxysmal nocturnal hemoglobinuria; EGR-1; TAXREB107

Contents

1. Introduction

1.1. Paroxysmal nocturnal hemoglobinuria

1.1.1. Clinical appearance

Paroxysmal nocturnal hemoglobinuria (PNH) was first described as a distinct clinical entity by Paul Strübing in 1882 (Strübung 1882). Through careful observation of clinical symptoms he recognized PNH as distinct from other hemolytic anemias and, in particular, being different from the hemolytic anemia due to cold hemolysis which was commonly found in cases of syphilis at that time. He noted that the hemolysis was intravascular and reasoned that it might be a result of intrinsic abnormality of the red cell membrane. About 50 years later, Ham demonstrated that the hemolysis in PNH is due to abnormal sensitivity of red cells to complement (Ham and Dingle 1939), which can be tested *in vitro* by acidification of autologous plasma activating especially the alternative complement cascade.

Paroxysmal nocturnal hemoglobinuria is an acquired blood disorder; its incidence is estimated at about one case in scores of hundred thousands of persons (Luzzatto et al. 1997; Bessler and Hillmen 1998). The diagnosis is most frequently in adults of age 30-50; however, PNH also occurs in the oldery and in children as young as 0,8 year (Ware et al. 1991; Hillmen et al. 1995; Socie et al. 1996). Patients with PNH classically have hemoglobinuria caused by intravascular hemolysis, suffer from thrombosis and have complications of bone marrow failure (Dacie and Lewis 1972). The severity of symptoms often varies, which often delays diagnosis.

The name of the disease was coined from hemoglobinuria, the passage of red or dark brown urine, although this symptom is found initially in only a

quarter of patients. The extent of hemolysis and the degree of anemia are highly variable from patient to patient.

Hemoglobinuria in patients with PNH is caused by intravascular lysis of red cells that are abnormally sensitive to complement attack. However, not all red cells have this increased sensitivity, since a variable proportion of them is normal. At the same time a proportion of other blood cells displays the same defect of the abnormal red cells, namely deficiency of all proteins bound to the cell membrane by a glycosylphosphatidylinositol (GPI)-anchor (Davitz et al. 1986). The residual normal blood cell population is often reduced in number, suggesting that it is derived from failing bone marrow. Many patients with PNH have evidence of deficient hematopoiesis, and the degree of bone marrow failure varies from subclinical cytopenia to the development of severe aplastic anemia (AA). Conversely, PNH has also been described in patients affected by aplastic anemia (Dameshek 1967; Lewis and Dacie 1967). The main causes of morbidity and mortality in PNH are venous thrombosis, which is appeared to occur in 40% of patients; and the results of intravascular hemolysis, which are associated with hemoglobinuria, abdominal pain and, not frequently, dysphagia. The main causes of death due to PNH are venous thrombosis and complications from progressive pancytopenia (Hillmen et al. 1995; Socie et al. 1996).

1.1.2. The cellular phenotype of PNH-cells

The hallmark of PNH blood cells in that they are deficient in all proteins that use a glycosylphosphatidylinositol (GPI)-anchor molecule (Davitz et al. 1986) (Figure 1). Today, at least 27 different GPI-linked proteins are known to be deficient on PNH blood cells. The lack of 2 of those molecules is sufficient for the diagnosis of PNH.

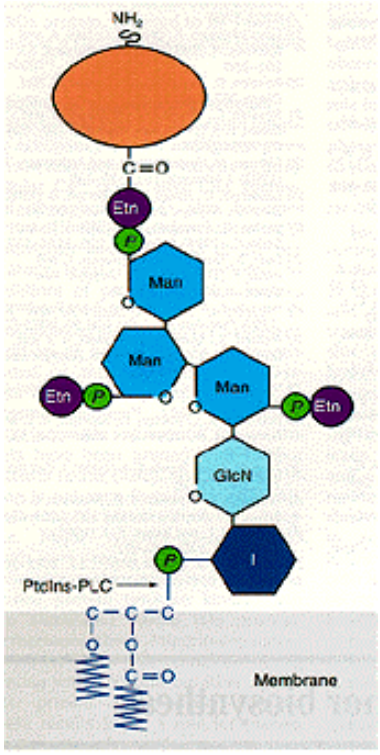

Figure 1: Structure of the GPI-anchor.

GPI-linked proteins on hematopoietic cells perform a multitude of functions. They serve as ecto-enzymes, accessory molecules for growth receptors, complement inhibitors, or adhesion molecules. CD55 (or DAF – decay accelerating factor) and CD59 (or MIRL – membrane inhibitor of reactive lysis) are complement regulatory proteins that inhibit complement activation on the cell surface (Nicholson-Weller et al. 1983; Holguin et al. 1989). The lack of these 2 proteins on red cells is responsible for their increased sensitivity toward complement and thus intravascular hemolysis and hemoglobinuria in PNH. Based on this observation the Ham test of PNH diagnosis was developed. More recently, analysis of blood cells by flow cytometry with monoclonal antibodies to GPI-linked surface molecules has been used for the diagnosis of PNH. When performed on granulocytes and erythrocytes this

type of analysis has a high sensitivity and is the preferable diagnostic test for the current time (Hall and Rosse 1996).

The biological role of GPI anchor is not fully understood. GPI-linked proteins can be released by specific phospholipases (GPI-PLS and GPI-PLD) (Low 2000). In thrypanosomes, GPI-PLC-controlled release of the variant specific glycoprotein, the main coat protein, enables the parasite to evade the host immune response. However, the functional role of GPI-PLC and GPI-PLD in human tissues remains elusive. Good lateral mobility, which is another characteristic of GPI-anchor, is likely to be relevant for many GPI-linked proteins that require the clustering of GPI-anchor molecules in the association with microdomains, or "rafts", enriched in glycophospholipids and cholesterol. The association with microdomains contributes to the specific surface distribution of GPI-linked proteins and aids in the recruitment of accessory molecules for the cell signaling (Horejsi et al. 1998).

GPI-anchored proteins are widely distributed among cell surface proteins in eukaryotic organisms and are highly conserved in all eukaryotic cells. In all species, the GPI anchor shares a common core region consisting of ethanolamine phosphate, 3 mannoses, glucosamine and inositol.

1.1.3. Complement-mediated hemolysis

For erythrocytes the function of two GPI-anchored proteins — CD55 and CD59 — is very important. They play a role in protection of the cell against lysis by activated complement, especially the alternative complement cascade. CD55 (DAF) destabilizes and inhibits the formation of C3-convertase. However, the most significant protective molecule is evidently CD59 (MIRL), a 20-kDa membrane glycoprotein discovered in 1989. CD59 designates its status as a cell-surface marker recognized by specific antibody. It binds to the C8 component of complement, thereby preventing C9 from fully binding, an

event that would initiate the polymerization of C9 into the toruslike structure required for the complement system to make a hole in the cellular membrane (Figure 2).

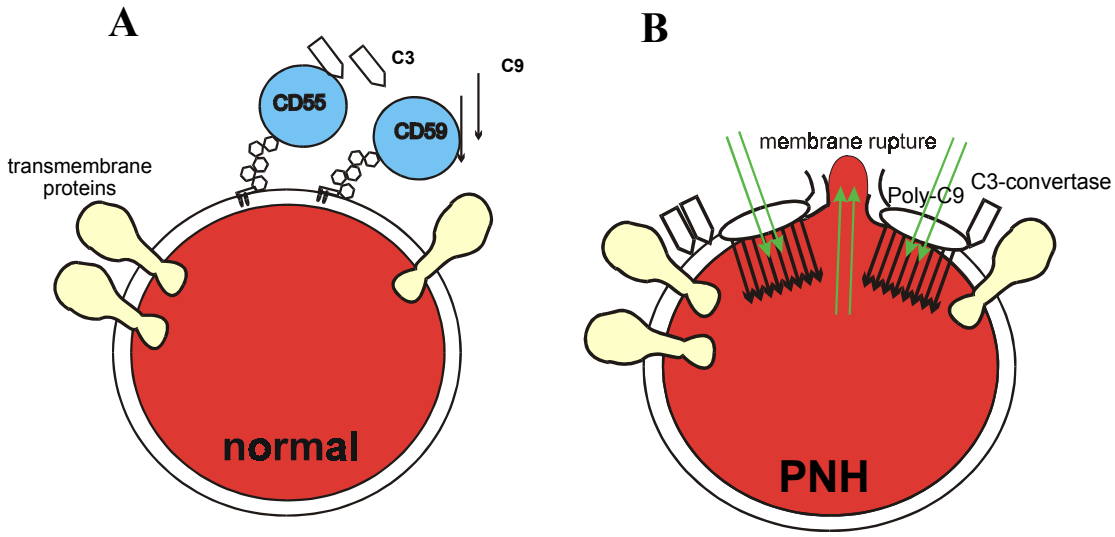

Figure 2: Schematic representation of complement-mediated lysis of GPI-anchor deficient erythrocytes in PNH. A: On the surface of normal cell CD55 inhibits the formation or destabilizes the C3 convertase and CD59 protects the membrane from attack by the C5-C9 complex. B: On the surface of GPI-deficient erythrocytes in PNH both proteins are missing. Therefore, membrane attack complex can be formed what leads to membrane rupture and cell lysis.

1.1.4. GPI-anchor biosynthesis and PIG-A gene

Biochemical analysis of GPI-anchor biosynthesis in cell line obtained from patients with PNH shown that the mature GPI anchor is not formed. In all cell lines tested, the block in the biosynthetic pathways always occurs in the first step when uridine diphospho-N-acetylglucosamine (UDP-Glc-NAc) is

transferred to phosphatidylinositol to form N-acetylglucosaminyl-phosphatidylinositol (Glc-NAc-PI) (Armstrong et al. 1992). At least 6 gene products are involved in this particular step; 4 of which are essential for GPI-anchor biosynthesis (Watanabe et al. 2000).

During the time when genes involved in GPI-anchor biosynthesis were unknown, cell lines deficient in GPI-linked proteins were obtained by *in vitro* mutagenesis. The mutant cell lines were grouped into 10 different complementation classes by using somatic cell fusion experiments. Fusion of cell lines derived from PNH patients with the mutant cell lines showed that all PNH cells tested belonged to complementation class A. This suggested that PNH cells and mutant cell lines from the complementation class A have the genetic defect (Armstrong et al. 1992; Takahashi et al. 1993).

In 1993, the gene defective in cell lines from complementation group A was cloned and accordingly was named *PIG-A*, which stands for phospatidyinositol glycan complementation class A (Miyata et al. 1993). When transfected into GPI-deficient cells from PNH patients, the *PIG-A* complementary DNA restored the expression of GPI-linked proteins on the cell surface (Takeda et al. 1993; Bessler et al. 1994). After the cloning of the *PIG-A* gene, mutations were identified in blood cells from patients with PNH, which finally confirmed that the *PIG-A* gene is the gene mutated in PNH (Miyata et al. 1994; Ostendorf et al. 1995; Luzzatto and Nafa 2000).

The *PIG-A* gene is approximately 17 kilobases (kb) long and has 6 exons. Exon 1 is noncoding, and exon 2 accommodates almost half of the *PIG-A* gene coding region (Bessler et al. 1994; Iida et al. 1994). Exon 5 contains sequences that have homology to the Glc-NAc-transferase of *Salmonella typhimurium* and glycosyltransferases in plants and thus might be the binding site of UDP-GlcNAc (Bessler et al. 1994). Exon 6 contains the transmembrane domain of *PIG-A*. The PIGA cDNA consists of 3589 base pairs (bp) with an open reading frame of 1452 bp encoding a putative protein of 484 amino acids (Miyata et al. 1994). The *PIG-A* gene maps to the short arm of the X chromosome at Xp22.1.

A nonfunctional processed pseudogene has been mapped to 12p21 (Bessler et al. 1994).

The PIG-A protein locates to the endoplasmic reticulum (ER). The amino terminus of the protein lies on the cytoplasmic side of the ER where the initial step of GPI-anchor synthesis takes place. The PIG-A protein is a subunit of the UDP-GlcNAc:PI-α-1, 6-GlcNAc-transferase complex, which is formed by at least 6 different protein subunits (PIG-A, PIG-H, PIG-C, GPI-1, dolichol-phosphate-mannose synthase (DPM-2), and PIG-P). PIG-A binds to PIG-H, GPI-1, DPM-2 and PIG-P, but is not stable associated with PIG-C (Hong et al. 1999; Watanabe et al. 2000). PIG-A, like PIG-H, PIG-C and PIG-P, is essential for the enzymatic activity of the glycosyltransferase. In contrast, GPI-1 and DPM-2 are not essential components of the transferase but enhance its activity (Watanabe et al. 2000).

To date, over 180 mutations have been published to be found in GPI-deficient blood cells from patients with PNH (Luzzatto and Nafa 2000). The majority of *PIG-A* gene mutations are frame shift mutations that predict an inactive PIG-A protein and a loss of glycosyltransferase activity. The mutations are distributed over the entire coding region with no obvious clustering. Only 16 mutations out of 174 were reported to be found in more than 1 PNH patient (Nishimura et al. 1999). There is some clustering of missense mutations over the coding region of exon 2. Some of these missense mutations have been shown to cause only a partial deficiency in GPI-linked proteins on the cell surface, indicating that the mutant glycosyltransferase has some residual activity (Bessler et al. 1994). One mutation (nucleotide 55 C→T, Ava I polymorphism) is not associated with the loss of GPI-linked proteins on the cell surface and, in contrast to all other *PIG-A* mutations, it is inherited in Mendelian fashion. All other mutations are presented only in the patient's GPI-deficient cells, but not in the patient's normal cells, which is consistent with the somatic nature of the *PIG-A* mutation and the clinical fact that PNH is an acquired disease.

The cloning of the *PIGA* gene and identified mutations have considerable impact on our understanding of the pathogenesis of PNH. The mutations explain the deficiency of GPI-linked proteins on the cell surface and the clinical symptoms of intravascular hemolysis and hemoglobinuria (Figure 3).

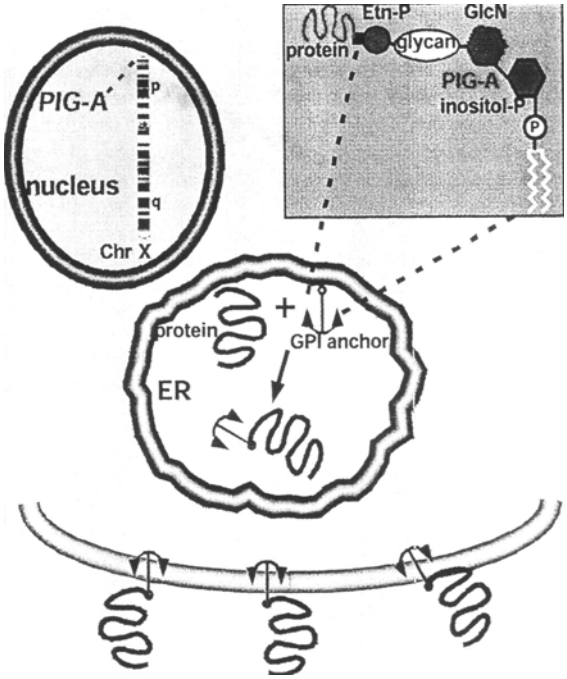

Figure 3: *PIG-A*, GPI-anchors, GPI-anchored proteins and PNH. PIG-A is a protein encoded by the X-linked gene *PIG-A*. PIG-A is a member of multi-subunit enzymatic complex which catalyzes in the endoplasmic reticulum (ER) the first step in the biosynthesis of GPI: the addition of acetylglucosamine (GlcN) to phosphatidylinositol (inositol-P). The synthesis of the GPI-anchor is completed by the serial addition of a glycan moiety consisting of three mannose molecules and a molecule of phosphoethanolamine (Eth-P), to which, through a transpeptidation reaction, proteins with appropriate carboxy-terminal amino acid motif are covalently attached. The GPI-protein becomes "anchored" to the lipid bilayer through GPI. In PNH, *PIG-A* has undergone somatic mutations within one or few HSCs, which are clonaly expanded. As a result, very few GPI-anchor molecules is synthesized, or none at all, leading to subsequent severe deficiency of GPI-linked proteins on the surface of the mutated HSCs and their progeny (from (Karadimitris and Luzzatto 2001)).

PIG-A gene is localized on the X chromosome and, therefore, it is subject to X chromosome inactivation in females (Keller et al. 1999). Therefore, a single mutation can cause the loss of GPI-linked proteins on both male and female somatic cells. Thus, in males the inactivation of the one *PIG-A* allele is sufficient to cause the loss of GPI-linked proteins on the cell surface. In females the inactivation of *PIG-A* allele when it is on the active X chromosome will lead to the loss of GPI-linked proteins on the cell surface (due to random X-inactivation this is expected to occur in about half of the cells). Therefore, in both male and female cells a single mutation (1 hit) is sufficient to inactivate PIG-A protein function and cause the loss of GPI-linked proteins on the cell surface. Moreover, all other genes involved in the GPI-anchor biosynthesis are autosomal. Therefore, the loss of GPI-anchored proteins would thus require 2 mutations, an event that is extremely unlikely to occur.

1.1.5. PNH as a consequence of the clonal expansion of GPI-deficient cells

1.1.5.1. PNH stem cells have an intrinsic growth advantage but PIG-A gene mutation by itself does not provide it

The clonal character of the GPI-deficient cells of a PNH patient has been initially shown by polymorphism analysis of the glucose-6-phosphat-dehydrogenase in the complement sensitive erythrocytes (Oni et al. 1970). Since in PNH GPI-deficient cells always coexist with at least a small fraction of cells with normal phenotype the hypothesis that PNH is an acquired clonal defect has been established (Rotoli et al. 1984). Recent studies of the topic show that PNH is not necessarily a monoclonal illness. At least in some patients several different clones with different *PIG-A* mutations contribute to the GPI-deficient cell fraction. Up to 4 different *PIG-A* gene mutations have

been identified to coexist in the bone marrow from PNH patients and give rise to independent PNH clones. Hereby, PNH rather needs to be understood as oligoclonal disease (Bessler et al. 1994; Parker 1996).

In colony-forming assay has been observed decreased hematopoietic capacity for bone marrow cells of PNH patients measured by CFU-E (Rotoli et al. 1982). This observation was the basis for the hypothesis that GPI-deficient cells shows not absolute but a relative growth advantage in the bone marrow of the PNH patients (Schubert et al. 1994; Luzzatto and Bessler 1996). More recently CD34+ cell numbers and the number *in vitro* cultivated long-term-culture-initiating-cells (LTCIC) from the bone marrow and the peripheral blood of normal donors and PNH patients were compared (Maciejewski et al. 1997). A clear difference of cell numbers for normal donors and PNH patients was observed, but not in the LTCIC formation from CD34+CD59+ and CD34+CD59- cells of the same PNH patient.

To check these theories further, there have been done some experiments in order to generate *PIG-A*-knockout mice. However, *PIG-A*-knockout animals were not capable to survive, because some of GPI-anchored surface molecules, particularly N-CAM, are essential for the neural development of mammalian (Schubert et al. 1994). Therefore, as a model of PNH a chimeric *PIG-A* knockout mouse was developed. The relative amount of GPI-deficient cells decreases in such chimeric *PIG-A*-knockout mice at a prolonged observation period (Kawagoe et al. 1996; Rosti et al. 1997).

In addition, GPI-deficient clones containing *PIG-A* mutations have even been detected in normal donors at trace amounts and without a tendency to expand (Luzzatto and Bessler 1996; Araten et al. 1999).

Taken together, these data suggest an intrinsic growth advantage of the PNH stem cell dominating the bone marrow and indicate that a *PIG-A* gene mutation by itself neither provides such an intrinsic growth advantage nor does it cause bone marrow failure. Indeed, in a neutral environment, the *PIG-A*- hematopoiesis is rather indifferent.

At the moment it is not clear why GPI-deficient cells in PNH are able to expand. Three hypotheses were offered as an attempt to explain such a clonal expansion despite the growth and activation disadvantage associated with lack of GPI-linked cell surface proteins.

1.1.5.2. Somatic cell selection and immunological attack escape in PNH

The first of these hypotheses is a clonal selection mediated by an immunological attack against the bone marrow stem cells from which GPI-deficient cells escape by an increased resistance to the action of cytotoxic cells (Young 1992). This hypothesis is based on the observation that within the aplastic anemia complete remission can be achieved by immunosuppressive therapy (Cosimi et al. 1982; Gluckman et al. 1982; Frickhofen et al. 1991). In addition, an expansion of GPI-deficient cells was observed for patients without hematological system illness, which were treated with the monoclonal antibody Campath-1H against GPI-linked CD52 (Hertenstein et al. 1995). The cytotoxic effect of the rat's antibody caused a selection in favor GPI-deficient lymphocytes and monocytes within patients, so that the GPI-deficient cells can expand.

The mechanism of bone marrow failure is still poorly understood but is believed to be immune mediated. Hypothesized escape mechanism of PNH cells toward this immune attack is unclear but is presumed to be mediated by one or more GPI-linked cell surface antigens. Recent reviews propose a variety of models that might explain the escape of PNH cells, but so far a clear implication of the role of any GPI-linked protein is lacking.

According to the immune selection theory proposed by the group of L.Luzzatto (Bessler et al. 1994) autoimmunity is primarily directed against either the GPI-anchor or a GPI-linked protein. Immune selection theory is supported by observation of a skewed T-cell repertoire in PNH patients that indicates the presence of specific T-cell clones recognizing the GPI-anchor or a

GPI-linked protein (Karadimitris et al. 2000). However, normal and GPI-deficient cells turned out to be equally sensitive to allogenic T lymphocytes (Karadimitris et al. 2000).

Recently 2 proteins, at least one of which is GPI-linked, have been identified in mice that may directly connect the lack of GPI-linked proteins to the immune escape mechanism. Both proteins, retinoic acid early inducible (REA-1) and H-60, bind the NKG2D receptor of natural killer (NK) lymphocytes (NK cells). Ligand binding of NKG2D leads to NK cell activation and NK-mediated cell death mainly by the perforin-granzyme pathway. Both ligands are relatively restricted in expression on normal adult mouse tissues but are upregulated on stressed cells. Thus, the human REA-1 and H-60 homologues (ULBOs, UL16 binding proteins) might be interesting candidate molecules that when expressed on hematopoietic stem cells in the early stages of bone marrow failure activate NK cells to kill the cell carrying them. In contrast, PNH stem cells fail to express these ligands and thus escape immune surveillance by NK cells.

1.1.5.3. Resistance to apoptosis

As an alternative to the immune escape it has been suggested that GPI-deficiency itself leads to an increased resistance to apoptosis (Brodsky et al. 1997). An increased rate of apoptosis has been observed in patients with aplastic anemia or myelodysplastic syndrome. If GPI-deficient cells only by absence of GPI-linked surface molecules would exhibit such a resistance clonal dominance of the GPI-deficient clone would result in the context of AA or MDS. However, such a resistance just due to GPI-deficiency could not be reproduced by other groups (Ware et al. 1998; Bastisch et al. 2000).

1.1.5.4. Appearance of additional alterations in cellular activating repertoire is necessary for PNH development

The third hypothesis based on the observation that paroxysmal nocturnal hemoglobinuria (PNH), aplastic anemia (AA) and myelodysplastic syndrome (MDS) are related diseases (Marsh and Geary 1991; Young and Maciejewski 2000). Therefore, clonal dominance can be caused by appearance of additional mutations in cellular activating genes in different parts of the bone marrow. This hypothesis is confirmed by the observation that early hematopoietic progenitor cells of PNH patients can grow *in vitro* without addition of exogenous cytokines in contrast to those of healthy donors (Issaragrisil et al. 1986). In addition, preferential hematopoiesis of PNH clone engrafted in SCID mice has been observed (Iwamoto et al. 1996).
Moreover, GPI-deficient cells characteristic for PNH are very often detectable in AA and MDS (Yamaguchi et al. 2000). It has been observed that genetic instability is involved in the pathogenesis of these hematopoietic stem cell disorders. Investigation in vivo mutation frequencies suggest that not only PIG-A but also other genes are hypermutable in PNH, AA and MDS, and that mutagenic pressure and/or gene instability can contribute to the pathogenesis of these disorders (Hattori et al. 1997).
Therefore, clonal expansion of GPI-deficient cells in PNH might be caused by additional gene alterations.

Which of these hypotheses will turn out at the end to be valid is not currently clear. It is also possible that a combination of both mechanisms leads to the expansion of the GPI-deficient clone. Besides, the genetic changes in the activation program of the cells could themselves lead to a secondary immunological reaction.

1.2. Aim of the study

Paroxysmal nocturnal hemoglobinuria is an acquired clonal defect of hematopoietic stem cells characterized by deficiency in GPI-anchored surface proteins. It is not yet known how GPI-deficient stem cells are able to expand within the bone marrow and contribute considerably to hematopoiesis. In PNH as well as in AA and MDS, genetic instability and increased mutation frequency have been detected. Therefore, a second event is very likely, such as additional mutations or differential gene regulation, leading to clonal expansion of GPI-deficient bone marrow stem cells in PNH.

The aim of the study was to investigate molecular bases of clonal expansion in PNH and identify genes differentially expressed in normal and GPI-deficient cells of PNH patients, analyze the genes found, confirm their differential expression and characterize it in PNH.

2. Materials and Methods

2.1. Materials

2.1.1. Chemicals and reagents

2.1.1.1. Fine chemicals

Chemicals and reagents were obtained from the companies Amersham, AppliChem, Becton-Dickinson, Biomol, Boehringer Mannheim, Gibco BRL, Difco, Fluka, Genomed, Hartmann, ICN, Merck, New England Biolabs, Pierce, Qiagen, Riedel de Haen, Roth, Serva, Sigma, Stratagene, Invitrogen. Other materials were obtained from Beckmann, Costar, Eppendorf, Eurogentec, Falcon, Kodak, Gilson, Sarstedt, Schleicher+Schuell and Whatman. Solutions for the cell culture were provided by Seromed/Biochrom.

2.1.1.2. Radiochemicals

[α^{32}P] dATP (3000 Ci/mmol)	Amersham, Braunschweig

2.1.2. Buffers and solutions

2.1.2.1 Buffers

TAE-buffer	40 mM Tris-Acetat 1 mM EDTA

10x TBE buffer	108 g Tris base (890 mM) 55 g boric acid (890 mM) 40 ml 0,5 M EDTA, pH 8,0
TE-buffer	10 mM Tris-HCl 1 mM EDTA pH 7,4
20x SSC buffer	3 M NaCl 0,3 M Na Citrate adj. with 1 M HCl to pH 7,0
5x MOPS buffer	0,1 M MOPS (pH 7,0) 40 mM sodium acetate 5 mM EDTA (pH 8,0) store up to 3 months at 4°C
PBS	140 mM NaCl 2,6 mM KCl 2 mM Na_2HPO_4 1,45 mM KH_2PO_4
6x gel loading buffer 1	0,25% Bromophenol blue 0,25% Xylene cyanol FF 15% Ficoll 4000 120 mM EDTA
6x gel loading buffer 2	0,25% Orange G 50% Glycerin

2.1.2.2. Other solutions and medium

LB-medium	1%(w/v) Trypton (AppliChem) 0,5%(w/v) Yeast extract (AppliChem) 1%(w/v) NaCl pH 7,0
Agar plates	LB-Medium 1,5%(w/v) Bacto-Agar (Difco) 100 µg/ml Ampicillin
SOC-medium	2%(w/v) Trypton (AppliChem) 0,5%(w/v) Yeast extract (AppliChem) 10 mM NaCl 2,5 mM KCl 10 mM $MgCl_2$ 20 mM Glucose pH 7,0

X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) stock solution (40 mg/ml):

Dissolve 400 mg X-Gal in 10 ml dimethylformamide.

Protect from light by storing in a brown bottle at -20°C.

Add to agar plates to final concentration 20-40µg/ml.

IPTG (isopropyl-β-D-thiogalactoside) stock solution (100 mM):

Dissolve 238 mg of IPTG in 10 ml deionized water.

Filter-sterilize and store in aliquots at -20°C.

Add to agar plates to final concentration 0,1 mM.

Ethidium bromide	10 mg/ml in distilled H_20
DMEM complete	DMEM 10 % (v/v) FCS (PAA Laboratories) 2 mM L-glutamine 100 U/ml Penicillin 100 µg/ml Streptomycin
RPMI 1640 complete	RPMI 1640 10 % (v/v) FCS 2 mM L-glutamine 100 U/ml Penicillin 100 µg/ml Streptomycin
Acrylamide/bisacrylamide 38:2 (w/w)	38 g acrylamide 2 g bisacrylamide H_2O to 100 ml Store ≤1 month at 4°C
0,5 M EDTA (pH 8,0)	186,1g $Na_2EDTA \times 2H_2O$ 700 ml H_2O adjust pH to 8,0 with 10 M NaOH (~50 ml) add H_2O to 1 liter

Stop/loading dye	in deionized formamide: 0.05% (w/v) bromphenol blue 0.05% (w/v) xylene cyanol 20 mM EDTA
4% denaturing acrylamide gel	
	75,6 g Urea 18 ml 38% acrylamide/ 2%bisacrylamide mix 18 ml 10x TBE buffer 180 µl TEMED 500 µl 10% (w/v) APS
Transfer solution for downward alkali blotting	
	3 M NaCl 8 mM NaOH (pH 11,4)
Neutralizing solution (0,2 M sodium phosphate buffer, pH 6,7-6,8)	
	46,3 ml of 1 M Na_2HPO_4 53,7 ml of 1 M NaH_2PO_4 H_2O to 500 ml
Stripping solution	1% SDS 0,1 x SSC 40 mM Tris-Cl, pH 7,5-7,8

Denaturation solution for FISH	70% formamide 20x SSC adjust pH to 7,0-8,0
Formamide wash solution	50% formamide 2x SSC adjust pH to 7,0-8,0

2.1.2.3. Molecular weight markers

100 bp DNA Ladder (New England BioLabs) with 1517, 1200, 1000, 900, 800, 700, 600, 500/517, 400, 300, 200, 100 bp fragments.

1 kb DNA Ladder (Gibco BRL) with 12216, 11198, 10180, 9162, 8144, 7126, 6108, 5090, 4072, 3054, 2036, 1636, 1018, 517/506, 396, 344, 298, 220, 201, 154, 134, 75 bp fragments.

λ DNA/Hind III (Gibco BRL) with 23130, 9416, 6557, 4361, 2322, 2027, 564 bp fragments.

2.1.3. Bacterial strains, cloning vectors and plasmids

2.1.3.1. Bacterial strains

XL1-Blue	Stratagene F´Tn*10* *pro*A$^+$B$^+$ *lac*I^q Δ(*lacZ*)M15/*rec*A1 *end*A1 *gyr*A96 (*Nal*^{r}) *thi hsd*R17 ($r_K^-m_K^+$) *sup*E44 *rel*A1 *lac*
INVαF´	Invitrogen F´*end*A1 *rec*A1 *hsd*R17 (r_k^-, m_k^+) *sup*E44 *thi*-1 *gyr*A96 *rel*A1 ϕ80*lacZ*ΔM15 Δ(*lacZ*YA-*arg*F)U16 λ$^-$
TOP10F´	Invitrogen F´ {*lac*I^q Tn*10* (Tet^R)} *mcr*A Δ (*mrr-hsd*RMS-*mcr*BC)ϕ80*lacZ*ΔM15 D*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (*Str*^{R}) *end*A1 *nup*G

2.1.3.2. Cloning vectors

pCR2.1	Invitrogen Vector pCR2.1 pCR2.1 contains the lacZ-alpha complementation fragment for blue-white color screening, ampicillin and kanamycin resistance genes for selection, and a versatile polylinker.
pcDNA3.1/Zeo	Invitrogen pcDNA3.1 vector is designed for high-level, constitutive expression in a variety of mammalian cell lines. It contains Zeocin® resistance gene for antibiotic selection.

2.1.3.3. RZPD clones

Clone ID	Gene	cloning vector
IMAGp998O245500Q2	STAT5a	pCMV-SPORT6
IMAGp998E234965Q2	STAT5b	pT7T3-Pac (mod)
IMAGp998F035797Q2	SRF	pT7T3-Pac (mod)

2.1.4. Oligonucleotides and primers

All oligonucleotides were synthesized at MWG-Biotech. The lyophilized primers were dissolved in distilled water at a stock concentration of 100 pmol/µl and stored at -20°C in aliquots.

Primer name	Primer sequence	Location
EGR-1 promoter1	CAA AGC CGG TCC TCT CTT CG	EGR-1 gene promoter
EGR-1 promoter2	AAC CAG CTC GGA CCG GAA TG	EGR-1 gene promoter
EGR-1 promoter3	CGG GCC ACT CCA AAT AAG GTG	EGR-1 gene promoter
EGR-1 promoter4	TGG TGG GCG AGT GAG GAA AG	EGR-1 gene promoter
EGR1_2527-2545	TTC TCA GTG TTC CCC GCG C	EGR-1 mRNA 5′-UTR
EGR1_a	GGA ACA GAG GAG TAC GTG GTG	EGR-1 mRNA CDS

EGR1_CDS-2521f	GCA CGC TTC TCA GTG TTC C	EGR-1 mRNA 5′-UTR
EGR1_CDS-5038r	TCT CCT CCT CCT GTC CTT TAA G	EGR-1 mRNA 3′-UTR
EGR1_forward	GGA GTC GCG AGA GAT CCA GC	EGR-1 gene promoter
EGR1_s	TTC CAG GTT CCC ATG ATC C	EGR-1 mRNA CDS
EGR1c-2906f	TTC AAC CCT CAG GCG GAC AC	EGR-1 mRNA CDS
EGR1c-3852r	GCT CAC TAG GCC ACT GAC CAA G	EGR-1 mRNA CDS
EGR1-CDS-2527forw	TTC TCA GTG TTC CCC GCG C	EGR-1 mRNA 5′-UTR
EGR1-CDS-2549forw	GCA TGT AAC CCG GCC AG	EGR-1 mRNA 5′-UTR
EGR1-CDS-5213rev	AAG AAC TTG GAC ATG GC	EGR-1 mRNA 3′-UTR
EGR-1promoter forw.	CGG TTC GCT CTC ACG GTC CC	EGR-1 gene promoter
EGR-1promoter rev.	GCT GGA TCT CTC GCG ACT CC	EGR-1 gene promoter
hum.actin, sense	CCA TGT ACG TTG CTA TCC AGG	Human actin mRNA CDS
hum.actin, antisense	TGC CAA TGG TGA TGA CCT G	Human actin mRNA CDS
TAXREB107, antisense	GGT CAG TCA CAA GTA ATA AGC	TAXREB107 mRNA CDS
TAXREB107, sense	CCA GAT ACT AAA GAG AAG AAA C	TAXREB107 mRNA CDS

rf180	TGG ACG TTG GCS	Random primer
rf187	TGC TGC AGG ACS	Random primer
rf24	GGA GAA GCT GCS	Random primer
rf328	GCA GCA TCC GGW	Random primer
rf417	ATG GCA ACG GCS	Random primer
rf522	GGC ACA TTG CGS	Random primer
rf567	CCA GAT GCC CGW	Random primer
rf688	AAG CTG CTC GCS	Random primer
rf942	ACG CCA TCG ACS	Random primer
rf95	TCG ATG CCG CTS	Random primer
T3	AAT TAA CCC TCA CTA AAG GG	Standard primer
M13reverse	CAG GAA ACA GCT ATG ACC	Standard primer
T7	GTA ATA CGA CTC ACT ATA GGG C	Standard primer

2.1.5. Eukariotic cell lines

Jurkat wild type (ATCC):
Human acute T cell leukemia cell line

HL 60 (ATCC):
Promyelocytic leukemia cell line

2.1.6. Enzymes

Enzymes used in this work were obtained from Roche Diagnostics GmbH, Gibco BRL, MBI Fermentas Molecular Biology GmbH, New England BioLabs, Pharmacia, Promega, Serva, Sigma-Aldrich Chemie GmbH and Stratagene GmbH.

2.1.7. Antibodies

Antibodies	Clone	Isotype	Conjugate	Producer
CD15	HI98	IgM, κ (Mouse)	PE	PharMingen GmbH
CD34	8G12	IgG1 (Mouse)	FITC	Becton Dickinson GmbH
CD34	581	IgG1, κ (Mouse)	PE	Becton Dickinson GmbH
CD34	AC136	IgG2a (Mouse)	FITC	Miltenyi Biotec
CD55	IA10	IgG2a, κ (Mouse)	Biotin, FITC, PE	PharMingen GmbH
HLA-ABC	W6/32	IgG2a (Mouse)	FITC	Serotec
Isotype Controls	DAK-GO1	IgG1 (Mouse)	FITC, PE	DAKO

2.1.8. Other materials

3MM filter paper	Whatman
15 ml and 50 ml tubes	Greiner
Nybond N+	Amersham Pharmacia Biotech
Petri dishes	Nunc
Pipet tips	Eppendorf
0,5; 1,5 and 2 ml tubes	Eppendorf
0,2 ml thinwall PCR tubes	Biozym
Cell culture flasks	Nunc

2.1.9. Kits

QIAprep Spin Miniprep Kit	QIAGEN
NucleoSpin® Plasmid isolation Kit	Macherey-Nagel GmbH
GFX™ PCR DNA band and Gel Purification Kit	Amersham Pharmacia Biotech
Original TA Cloning® Kit	Invitrogen BV
TOPO TA Cloning® Kit	Invitrogen BV
TRizolTM Total RNA Isolation Reagent	GibcoBRL
RNeasy Mini Kit	QIAGEN
RadPrime Labeling System	GibcoBRL
QIAamp DNA Blood Mini Kit	QIAGEN
Atlas pure Total RNA Labeling System	Clontech
ExpressHyb buffer	Clontech
Atlas *Hematology* cDNA array	Clontech

2.1.10. Laboratory equipment

1214 Rackbeta liquid scintillation counter (LKB)

2219 Multitemp II thermostatic circulator (LKB)

Biofuge 13 (Heraeus)

Cell culture incubators (Heraeus)

Centrifuge 5415 (Eppendorf)

Centrifuge, model J2-21, rotor JA14 and JA17 (Beckmann)

Centrifuge, model J-6B (Beckmann)

Centrifuge, model J6-MC (Beckmann)

Diavert fluorescence microscope (Leitz)

Diavert light microscope (Leitz)

Digital-pH-meter (Knick)

EasijectTM plus electroporation (Eurogentec)

Eclipse TE 300 fluorescence microscope (Nikon)

FACScalibur with CellQuest software (Becton Dickinson)

G24 environmental incubator shaker (New Brunswick Scientific Co. Inc.)

Gel Doc 1000 (Bio-Rad Laboratories GmbH)

GFL water bath (H. Juergens & Co.)

Horizontal electrophoresis (Bio-Rad Laboratories GmbH and Pharmacia)

Laminar air flow class 100 (Gelaire)

Magnetic cell separator, MACS (Miltenyi Biotec)

Megafuge 2.0 R (Heraeus)

Minifuge T (Heraeus)

PhosphorImager FujiFilm BAS1500 (Fuji)

SpeedVac plus SC 110A (Savant)

Sterile GARD hood (Baker Company, Inc.)

Thermocycler varius V45 (Landgraf)

T3 Thermocycler (Biometra)

Thermomagnetic stirrer, MR 2002 (Heidolph Instruments)

Thermomixer 5436 (Eppendorf)

Thermostat 5320 (Eppendorf)

UV-VIS Spectrophotometer, UV-1202 (Shimadzu Deutschland GmbH)

Vortex Genie 2TM (Bender and Hobein AG)

2.2. Methods

2.2.1. DNA-Modification

For modifying DNA, reactions with restriction enzymes, T4 DNA polymerase, alkaline phosphatase and T4 DNA ligase, were performed in accordance with the suppliers' instructions.

2.2.2. Plasmid isolation

For isolation of plasmids we used QIAprep Spin Miniprep Kit or NucleoSpin® Plasmid Kit.

The two methods are identical and designed for rapid and small-scale (20-40 μg) preparation of highly pure plasmid DNA (< 10 kb) from 1-8 ml of overnight *E. coli* culture. The mini-preparation of plasmid was carried out following the manufacturer's instructions. Briefly, the bacteria pellet was resuspended in RNase A-containing buffer 1 and *E. coli* host cells were lysed by SDS/alkali containing buffer (buffer 2). Buffer 3 was added to neutralize the resulting lysate and create appropriate conditions for binding of plasmid DNA to the silica membrane in QIAprep spin or NucleoSpin plasmid column. After following centrifugation step, the clear supernatant without SDS precipitate and cell debris was loaded onto QIAprep spin or NucleoSpin plasmid column. If host strains with high nuclease levels were used, a washing step with buffer PB (QIAprep Spin Miniprep Kit) or AW (NucleoSpin® Plasmid Kit) was performed. Washing with ethanol-containing buffer removed salts, metabolites and soluble macromolecular cellular components. Pure plasmid DNA was finally eluted with 50 μl distilled water and stored at -20°C.

2.2.3. RNA isolation

Total cellular RNA was extracted using TRIzol reagent in accordance with the recommendations of the manufacturer (GibcoBRL). Quality of the every RNA preparation was monitored by the A_{260} / A_{280} ratio and agarose-formaldehyde gel.

2.2.4. Genomic DNA isolation

Genomic DNA was isolated from whole blood by QIAamp DNA Blood Kit in accordance with the recommendations of the manufacturer (QIAGEN).

2.2.5. Polymerase chain reaction (PCR)

A standard PCR reaction was held as follows: 30 µl end reaction volume (filled up with dH_2O) with 100-300 ng genomic DNA or 20 ng plasmid DNA, 20-100 pmol of each primer, 200 µM dNTPs, 0,3 U Taq polymerase Gold and 1x buffer (including 1.5 mM $MgCl_2$) of polymerase supplier.
DNA was initially denatured at 95°C and incubated for 10 min in order to activate Taq polimerase Gold. PCR was carried out with 25 - 35 cycles for each 45 sec at 95°C; 45 sec at annealing temperature and 45 sec - 2 min extension at 72°C, depending on the length of DNA molecule to amplify. Final extension step was performed at 72°C for 5 – 15 min.

2.2.6. RT-PCR

2.2.6.1. First strand cDNA synthesis

In a sterile RNase-free microcentrifuge tube, 0.5 µg (1 µl) oligo(dT)$_{15}$ primer (Promega) was added to 1-5 µg of total RNA (treated or not with DNase I, RNase-free) in a total volume of 10 µl nuclease-free water. The tube was heated to 70°C for 10 min and immediately cooled on ice to prevent RNA renaturation followed by briefly centrifugation to collect the fluid at the bottom of the tube. The following components were added to the primer/template mixture in the following order:

5 X M-MLV reaction buffer	5 µl
10 mM dNTP´s (Promega)	1.25 µl
25 U rRNasin® ribonuclease inhibitor (Promega)	0.6 µl
nuclease-free water	7.15 µl
200 U M-MLV reverse transcriptase (Promega)	1 µl

The reaction mixture was incubated for 60 min at 37°C. M-MLV enzyme was inactivated by incubation at 95°C for 3 min and the tube was cooled on ice. First strand cDNA was stored at -80°C or the reverse transcriptase-polymerase chain reaction (RT-PCR) was immediately performed.

2.2.6.2. Reverse transcriptase-polymerase chain reaction

PCR reaction was held as follows: 5 µl of first strand cDNA from RT-step, 50 pmol of each primer, 200 µM dNTPs, 0,3 U Taq polymerase Gold and 1x buffer (including 1.5 mM $MgCl_2$) of polymerase supplier in 30 µl end reaction volume (filled up with dH_2O).

DNA was initially denatured at 95°C and incubated for 10min in order to activate Taq polimerase Gold. PCR was carried out with 25 - 35 cycles for each 45 sec at 95°C; 45 sec at annealing temperature and 45 sec - 2 min extension at 72°C, depending on the length of DNA molecule to amplify. Final extension step was performed at 72°C for 5 – 15 min.

2.2.7. Purification of PCR products and DNA fragments from gel bands

PCR products and DNA fragments from gel bands were purified with GFX MicroSpin columns following the instructions of the supplier (GibcoBRL). Briefly, 300 mg (the maximum weight that can processed with this procedure) of gel slice was dissolved in 300 µl capture buffer, mixed vigorously by vortexing and incubated at 60°C (5-15 min) until the agarose was completely dissolved. The sample was passed through the GFX column to capture the DNA onto the glass fiber matrix. Matrix-bound DNA was washed with an ethanol-containing buffer to remove any contamination. DNA was eluted from the column in a volume of 50 µl dH_2O.

2.2.8. Determination of nucleic acids concentration

To determine the concentration and purity of the isolated RNA and plasmid DNA, the absorption of diluted solution was measured by UV spectrophotometer at 260 nm (and at 280 nm in case of RNA). The dilution was performed to make the absorption between 0.1 and 1.0 OD.

Concentrations of nucleic acids were calculated as follows:

RNA concentration (µg/µl) = $A_{260} \times$ dilution factor $\times$ 40 /1000

DNA concentration (µg/µl) = A_{260} × dilution factor × 50 /1000
Purity of RNA was determined by $A_{260/}$ A_{280} ratio.

2.2.9. Precipitation of nucleic acids

To 1 volume of DNA or RNA solution 0,1 volume of 3 M NaAc (pH 5,2) and 2.5 volumes of Ethanol (or 0,7 volumes of Isopropanol) were added and mixed. After storing for at least 30 min at –20°C the mixture was centrifuged for 15 min with maximum speed. After discarding the supernatant the pellet was washed twice with 70% Ethanol and centrifuged for 5 min with maximum speed.
The supernatant was discarded and nucleic acid pellet was air dried for 10 min, resuspended in dH_2O and stored at -20°C.

2.2.10. Electrophoretic separation of nucleic acids

2.2.10.1. Electrophoresis of DNA in agarose gel

0.7%-1.2% (w/v) agarose gels containing 0,1 µg/ml ethidium bromide were used for analysis of plasmid DNA and PCR products. 1x TAE-buffer was used as gel and electrophoresis buffer. DNA samples were loaded into the gels in 1x DNA loading buffer 1 or 2 and run at 10 V/cm of the gel. Generally samples were run in parallel with a DNA molecular weight marker. The samples were visualized by Gel Doc 1000 (Bio-Rad) system and analyzed by molecular analysis software (Bio-Rad).

2.2.10.2. Electrophoresis of DNA in acrylamide gel

Electrophoresis in 4% denaturing acrylamide gel was used to resolve fragments, obtained from PCR-based RNA fingerprinting.
DNA sample/ stop-loading dye mixture was loaded into the gel. Gel was run in TBE buffer at a constant power 75 W and temperature kept below 55°C until xylene-cyanol reaches the bottom of the gel. The gel was transferred onto 3MM filter paper, vacuum dried and exposed overnight to Kodak MS film.

2.2.10.3. Electrophoresis of RNA in formaldehyde/agarose gel

RNA samples were mixed with 3-5 volume of RNA loading buffer (Sigma), denatured for 15 min at 65°C and briefly centrifuged before loading. The samples were separated in formaldehyde-agarose gels (1,2% (w/v) agarose, 1x MOPS buffer, 2,2 M formaldehyde). Gels were run at 5 V/cm in 1x MOPS buffer at 4°C.

2.2.11. Downward alkaline blotting of RNA

Downward alkaline capillary transfer for RNA blotting was performed as described in (Chomczynski 1992). Briefly, denaturing gel containing RNA samples was placed onto the bottom base formed by 2-3-cm-high stack of paper towels, several blotting papers and nylon membrane (Nybond N+, Amersham) well soaked in distilled water. Than the gel was covered with three shits of blotting paper and two sheets of blotting paper forming a connection (bridge) between the gel stack and tray containing the transfer solution. After 1,5-2 hour of transfer, the membrane was washed in

neutralizing solution, put between 2 Whatman sheets, air-dried overnight and baked in an oven for 20 minutes at 80°C.

2.2.12. Northern blot analysis

For Northern blot analysis, samples (2-10 μg) of total RNA were resolved by electrophoresis on 1,2% agarose, 2,2 M formaldehyde gels and blotted onto nylon membranes (Hybond N+, Amersham) using downward alkaline capillary transfer (Chomczynski 1992). Filters were hybridized in ExpressHyb solution (Clontech) following the manufacturer's instructions. Filters were visualized by autoradiography, than exposed to the storage phosphor screens and quantitated by PhosphorImager (FujiFilm BAS1500). Obtained images were analyzed using AIDA software. The intensity of signals was normalized to beta-actin as a housekeeping gene.

2.2.13. Cloning

Cloning of gel purified DNA fragments and PCR products into pCR®2.1 vector was performed with T4 DNA ligase. The Original TA Cloning® Kit contains linearized pCR®2.1 vector and provides a quick, one-step cloning strategy for the direct insertion of a PCR product into plasmid vector. The linearized vector has single 3`deoxythymidine (T) residues. The ligation reaction was performed with 1:1 or 1:3 (vector:insert) ratio in a 10 μl volume as follows:

Gel purified PCR product or DNA fragment	X µl
10 × ligation buffer	1 µl
Vector	2 µl
Sterile water	to a total volume of 9 µl
T4 DNA ligase (4 units)	1 µl

The ligation reactions were incubated overnight at 14°C. Than they were stored at -20°C until ready for transformation.

2.2.14. Transformation of chemocompetent E. coli

100 µl of competent E.coli cells were added to an ice-cold DNA suspension (50-100 ng DNA dissolved in 10 µl dH2O).
The mixture was incubated for 30 min on ice, then heat shocked for 90 sec at 42°C and afterwards cooled down on ice for 2 min. After adding 1 ml of SOC media, suspension was incubated being shacked for 45 min at 37°C. Aliquots of 100 - 500 µl were plated on appropriate selection media and incubated overnight at 37°C.

2.2.15. DNA sequencing

DNA sequencing was performed by MWG-Biotech sequencing service using the M13 reverse primer and T7 primer.

2.2.16. Eukariotic cell culture

2.2.16.1. General cell culture

Generally, adherent and suspension cells were grown at 37°C and 5% CO_2 in incubator. The saturated cultures were splited every 3 days. Cell density 10^5 – 10^6 cells/ml was maintained. To split and passage adherent cells, the cells were gently rinsed with PBS and trypsinized until the cells were easily detached. Trypsinization was quenched with medium prior to subculturing in fresh medium.

2.2.16.2. Cell counting

Suspension and trypsinized viable cell counts were performed using a Neubauer chamber (Hawksley & Sons Limited) with at least 200 cells being counted per sample. Cell viability was determined using trypan blue exclusion (Sigma-Aldrich Chemie GmbH).

2.2.16.3. Cells freezing down

To freeze suspension and trypsinized cells, the cells were centrifuged at 500xg for 5 min and the media was discarded. One ml of cold freezing solution (90% heat-inactivated fetal calf serum, 10% DMSO) per 10^6 cells was added immediately and the cells were transferred to a cold 2 ml cryogenic vial. After that the vial was placed overnight at -70°C and transferred to liquid nitrogen on the following day.

2.2.16.4. Cells thawing

To thaw the frozen cells, one vial was removed from liquid nitrogen and thawed rapidly at 37°C. 1 ml of medium was added immediately to the vial and the cells were gently transferred to a 15 ml sterile conical screw cap tube. Then 10 ml of medium was gently added and the suspension was mixed by inverting. After centrifugation at 500xg for 5 min the supernatant was removed, cells were resuspended in 10 ml of medium (10^5-10^6 cells/ml) and cultured in 25 cm^2 tissue culture flasks with filter cap at 37°C and 5% CO_2.

2.2.17. Generation of T-cell lines from patients with PNH

T-cell lines were generated as described in detail previously (Ostendorf et al. 1995). Briefly, cells were plated, at 1000 cells/well, onto a feeder layer of irradiated (5000 cGy) allogeneic PBL and EBV-transformed B-lymphoblastoid cells (Laz509). Colonies were expanded at 1000 cells/well in culture medium containing 10% lymphocyte-conditioned medium. The polyclonal T-cell lines, either normal or deficient for GPI-anchored proteins, were generated from CD48+ and CD48- sorted PBL of patients with PNH. After the first round of expansion, contaminating NK cells were depleted by using CD16 mAb B73.1 and the CD56mAb anti-Leu-19, followed by magnetic separation by using sheep anti-mouse antibody-loaded magnetic beads (Dynabeads; Dynal, Hamburg, Germany). A similar procedure with the use of CD48 antibodies MEM-102 was performed in order to remove contaminating CD48+ from CD48- T-cell lines. Re-analysis revealed a purity of CD3+ and CD48+ or CD48- cells of >95% in each experiment.

2.2.18. Isolation of mononuclear cells and granulocytes

Heparinized blood samples were obtained either from normal adult donors or from patients with PNH or other hematological diseases, and then centrifuged for 8 minutes at 800 rpm to collect plasma. After taking off plasma, blood cell pellet was mixed with an equal volume of RPMI 1640 medium and fractionated by centrifugation over Ficoll-Hypaque (Biochrom KG, Berlin, Germany). Mononuclear cells were harvested from the interphase of Ficoll density gradient. Granulocytes from the Ficoll gradient pellet were separated from erythrocytes by sedimentation in Gelafundin 4% (B.Braun Melsungen AG) followed by the hypotonic lysis and washing. The isolated granulocyte fraction was subjected to flow cytometry analysis to exclude possible cellular contaminations, i.e. with monocytes.

2.2.19. PCR-based RNA fingerprinting

Modified arbitrarily primed PCR fingerprinting of RNA (Welsh et al. 1992) was conducted as follows. A reverse transcription reaction was carried out using oligo$(dT)_{15}$ primer on DNase I treated total RNA. Each of radioactively labeled PCR reactions was performed from 5 µl of RT-reaction in a 50-µl final volume with one of 10 computer designed arbitrary 12-mer primers (final concentration 4 µM) (Consalez et al. 1999). In order to control the RNA amplification procedure and maximize efficiency of fingerprinting, all of RT- and PCR-reactions were carried out in duplicate. Amplified products were separated on 4% denaturing acrylamide gel and visualized by autoradiography. Differentially displayed bands were cut out of gel, electroeluted in TAE buffer and precipitated. The bands were reamplified using the same 12-mer primers, cloned into pCR2.1 vector (Invitrogen),

sequenced and subjected to further analysis. In order to preliminary screen obtained clones plasmids were blotted onto nylon membranes and hybridized with random primer labeled PCR mixture from the previous step.

2.2.20. cDNA array

Atlas membranes with 406 known genes (Clontech) were used. Radioactive cDNA probes were synthesized with Atlas pure Total RNA Labeling System (Clontech) on the base of RNA from 2 independent sets of GPI-positive and GPI-negative clones (CD48+ and CD48- T-lymphocyte cell lines from the same PNH patient) and hybridization was performed according to the manufacturer's recommendations. The signals were controlled by the signals of housekeeping genes and visually analyzed. Only those genes with strong signal differences were preliminarily considered as modulated.

2.2.21. Bioinformatics analysis

All obtained gene fragments were sequenced and searched against GenBank, dbEST and SwissProt databases through the BLAST server. The sequences were considered to be a part of known genes if they have 98% or more homology over at least a 200-bp DNA sequence in BLAST search.
For the gene analysis we used PubMed, GenBank, Entres, Locuslink, OMIM, UniGene databases and search engines.

2.2.22. Flow cytometric analysis (FACS)

Cells of interest (~10^5) were washed twice with ice-cold PBS and resuspended in 200 µl of staining buffer (PBS/0.2 % BSA) in 96-well plates. Fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-marked mAbs in appropriate concentrations were added to cell suspension following the instructions of supplier. After 30 min of incubation at 4°C and two washes with PBS cells were ready for incubation with secondary antibody or for phenotypic analysis on flow cytometer (FACScalibur with CellQuest software, Becton Dickinson). Nonspecific binding was blocked by FcR blocking reagent (Human IgG, Miltenyi Biotec). Appropriate conjugate isotype-matched Abs served as controls. Ten thousand cells from each sample were analyzed on flow cytometer. Data presentation was performed using WinMDI version 2.8 software.

2.2.23. In vitro stimulation of granulocytes

Granulocytes were suspended to 2×10^6 cells/ml in RPMI 1640 medium and incubated with 0,1 µg/ml G-CSF and with 0,1 µg/ml GM-CSF (TeBu) for 30 minutes, 1 hour, 1,5 hours and 2 hours at 37°C.

Granulocytes were obtained from normal donor and incubated (1×10^7 cells/ml) in plasma of normal donors and PNH patients for 0,5; 1,5; 2 and 4 hours at 37°C.

2.2.24. Fluorescence in situ hybridization analysis (FISH)

FISH was performed on granulocytes according to the instructions of the supplier. Briefly, up to 2×10^7 freshly isolated granulocytes were incubated in ice cold 0,075 M KCl for 10 min at room temperature. After 10 min centrifugation at 1200 rpm cell pellet was carefully resuspended in 10 ml of acetic acid/methanol (1:3) mix and incubated at room temperature for 25 min for fixation. Cells were stored at -20°C until ready for FISH.

100 µl of cells (density of 2×10^7 cells/ml) were pipetted in a drop onto cold glass slide and dried at room temperature. After denaturation at 73±1°C (denaturation solution) for 5 min glass slides were subsequently dehydrated in 70%, 85% and 100% ethanol for 1 min each and dried. Just before hybridization 10 µl of the LSI 5q EGR1 SO/ D5S23 SG probe was denatured at 73°C for 5 min and stored at 45-50°C. Glass slides were prewarmed at 45-50°C for 2 min and 10 µl of the probe was pipetted on the target area of each slide. Hybridization was performed at 37°C overnight, and subsequent washes were done at 46±1°C as follows: 3 times in 50% formamide/2×SSC for 10 min each, 2×SSC for 10 min, 2×SSC/0,1% NP-40 for 5 min. To visualize hybridization slides dried in darkness and 10 µl of counterstain (DAPI 150ng/ml) to the target area of the slide and coverslip were applied.

After hybridization the mean number of signals scored for each sample after counting 200 nuclei.

3. Results

In this work several genes differentially expressed in GPI-positive and GPI-negative cells of PNH patients were identified. In further studies expression of two genes was investigated, confirmed and characterized in PNH.

3.1. Identification of genes differentially expressed in GPI-positive and GPI-negative cells in PNH

In order to reveal differentially regulated genes responsible for the growth advantage and clonal expansion of the GPI-deficient clone in PNH, we have employed combination of two different approaches: the RNA fingerprinting technique and cDNA array hybridization. cDNA array hybridization technique allowed us to compare expression of hundreds of previously characterized genes at the same time. PCR-based RNA fingerprinting is powerful method to analyze expression not only known genes but also novel ones.

3.1.1. Generation of GPI-positive and GPI-negative PNH clones

As a model of GPI-positive and GPI-negative PNH clones we used the CD48+ and CD48- T-lymphocyte cell lines from the same PNH patient (A.P.) generated as described in *Methods*. Important that these cell lines have been obtained without any immortalization step (virus-transformation, *etc*.). The purity of GPI-negative and GPI-positive clones was monitored by flow cytometric analysis for every experiment (Figure 4). Cells have been used for further analysis only when purity more than 95% was detected.

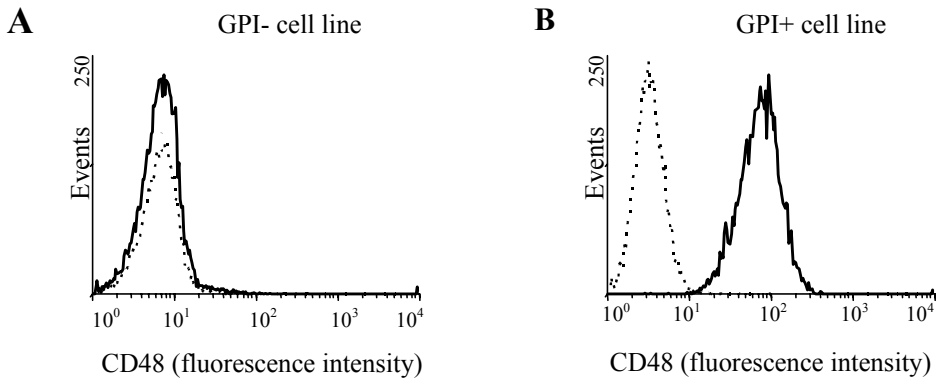

Figure 4: CD48+ and CD48- T-lymphocyte cell lines from the same PNH patient used as a model of GPI-positive and GPI-negative PNH clones. Results of flow cytometric analysis for the surface expression of CD48 are shown. CD48 fluorescence is presented as regular line, dashed line indicates isotope control.

Both employed techniques – the RNA fingerprinting technique and cDNA array hybridization analysis – require RNA as a starting material. Therefore, we isolated total cellular RNA from of GPI-positive and GPI-negative PNH cell lines. Then the total RNA was treated with DNase I to remove any tracks of genomic DNA to enhance result of gene expression profiling. The quality of RNA was monitored by agarose/formaldehyde denaturing electrophoresis and by ratio of optic density A_{260} to A_{280}. We used RNA in further experiments only when 28S rRNA band was approximately two fold brighter than 18S rRNA band and A_{260}/A_{280} ratio was higher than 1,8.

3.1.2. Atlas cDNA expression array hybridization

Analysis of gene expression can be accomplished using PT-PCR, RNase protection assays, or Northern blot analysis, but these methods focus on only

a few genes at a time. A more promising approach for analyzing multiple genes simultaneously is the hybridization of entire cDNA populations to nucleic acid arrays.

In this study we used human Atlas *Hematology* cDNA Expression Array from Clontech. It includes 406 known genes shown to play a role in human hematopoiesis and belonged to different functional classes.

Radioactive cDNA probes were synthesized with Atlas pure Total RNA Labeling System (Clontech) on the base of RNA from 2 independent sets of GPI-positive and GPI-negative clones (CD48+ and CD48- T-lymphocyte cell lines from the same PNH patient). Hybridizations with separate Atlas *Hematology* cDNA arrays were performed simultaneous for both analyzing probes according to the manufacturer's recommendations. The images were obtained by autoradiography (Figure 5). The differential signals were controlled by the signals of housekeeping genes and visually analyzed. Only those genes with strong signal differences were preliminarily considered as modulated.

3.1.3. PCR-based differential screening – RNA fingerprinting

The PCR-based differential screening – RNA fingerprinting can be successfully applied not only to identify differentially expressed genes, but also to isolate and characterize novel ones. It is based on the reverse transcription of mRNAs and PCR using arbitrary primers.

In this study we used modified arbitrarily primed PCR fingerprinting of RNA with 10 computer designed arbitrary 12-mer primers (described in detail in *Materials and Methods*). All of RT- and PCR-reactions were carried out in duplicate in order to control the RNA amplification procedure and maximize efficiency of fingerprinting. Amplified products were separated on denaturing acrylamide gel and visualized by autoradiography (Figure 6).

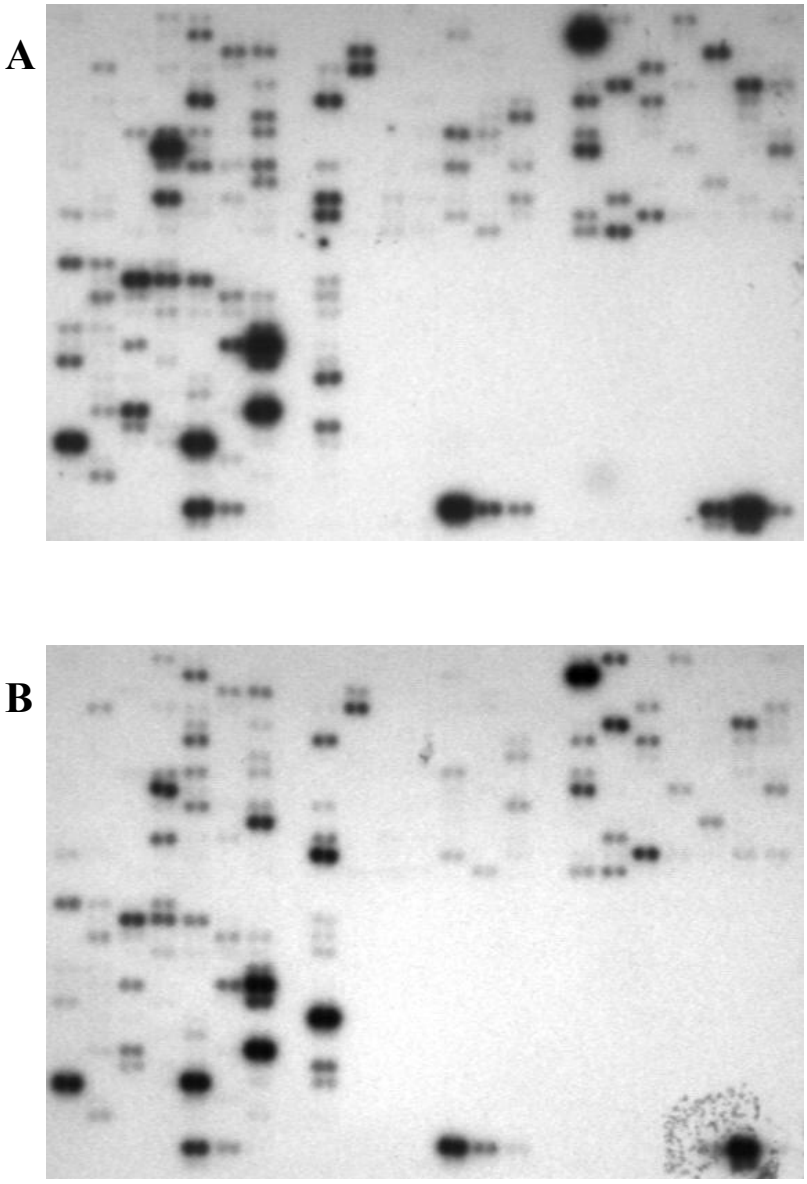

Figure 5: Gene expression profiling using *Hematology* Atlas cDNA Expression Array. ^{32}P-labelled cDNA probes were prepared from 25 µg of total RNA isolated from CD48+ and CD48- T-lymphocyte cell lines from the same PNH patient (panel A and B corresponding). The probes were simultaneously hybridized to separate Atlas *Hematology* Array membranes. Results were analyzed by autoradiography.

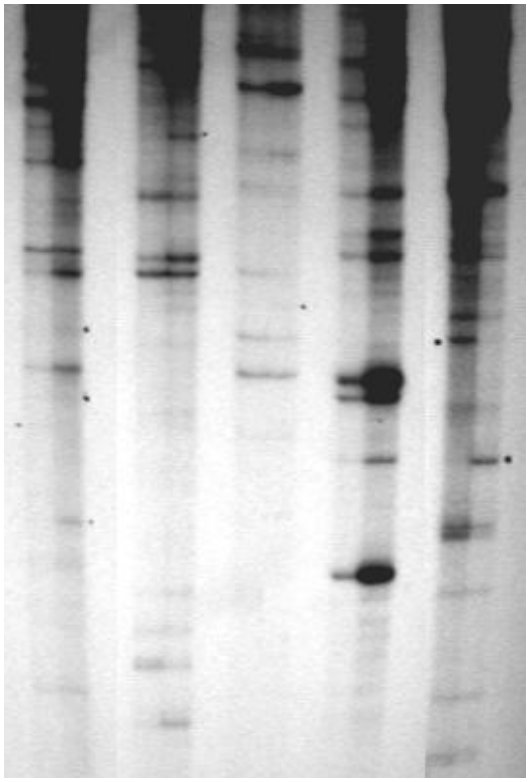

Figure 6: PCR-based differential screening - RNA-fingerprinting. Total RNA was isolated from GPI-positive and GPI-negative PNH lines, reverse transcribed and subjected to amplification of coding region fragments with computer-designed arbitrary primers. Obtained PCR-products were resolved by polyacrylamide gel. Part of the gel is presented.

Differentially displayed bands were cut out of the gel and electroeluted. The PCR fragments were reamplified using the same 12-mer primers and screened for probe specificity as described in *Methods.* After screening all positive PCR products were cloned, sequenced and subjected to further analysis.

3.1.4. Genes found to be differentially expressed in GPI-deficient PNH cells.

Finally, a number of candidate genes and cDNA clones were identified as being differentially expressed in GPI-deficient cells. cDNA array

hybridization analysis revealed 15 out of 406 genes, involved in human hematopoiesis, exhibiting a significant difference of expression levels (Table 1). Among these 15 genes, 7 out of them were up-regulated in GPI-deficient cells, and 8 displayed down-regulated signals.

PCR-based RNA fingerprinting gave rise to a total of 11 sequences with confirmed differential regulation. Sequence analysis of these clones led to identification of 4 known genes, 4 novel sequence fragments, and 3 were matched repetitive elements (Table 1). Expression of identified genes was further tested by RT-PCR and Northern blot analysis.

Name of protein/gene	GenBank accession #	Difference in expression	Name of protein/gene	GenBank accession #	Difference in expression
CALLA; CD10 antigen	X07166	–	MLR3; BL-AC/P26; AIM	L07555	– – –
TAXREB107; C140	X69391	+ + +	GZMK; fragmentin 3	U35237	+ + +
ID2	M97796	–	chromosome 9	NT_008387.2	+
DEK protein	X64229	–	Chromosome 14	AL117694	–
TOP1	J03250	+	Chromosome 9, Alu repetitive element	NT_008528.2	+
SMMHC; MYH11	X69292	+ + +	P2X	XM_008509	+
CREB1	M34356	–	CDNA DKFZp434D2328	AL133087	+ + +
BSG; EMMPRIN	L20471	– – –	Chromosome 6	NT_023346.2	–
LYT1	X04391	– – –	L1 repetitive element, chromosome 6, 7, 16	AC090497	+
MLLT6; AF17	U07932	+ + +	EGR1	NM_001964.1	+ + +
STAT1	M97935	+	HuMCM5	NM_006739.1	+ + +
ITGA4; CD49D	L12002; X16983	– – –	Chromosome 10, Alu repetitive element	NT_008921.2	–
CD19; B4	M21097	+ + +	Chromosome 9	NT_008387.2	+

Table 1: Genes, found to be differentially expressed in GPI-deficient and normal cells of PNH-patients. Two different approaches, the RNA fingerprinting technique and cDNA array hybridization, revealed 26 genes differentially expressed in PNH. Plus indicates strong (+ + +) to week (+) upregulation of the gene in GPI-deficient cells in comparison to normal cells level, minus indicates strong (– – –) to week (-) downregulation of the gene in GPI-deficient cells in comparison to normal cells level.

All genes found to be differentially expressed in GPI-negative and GPI-positive cells of PNH patients were subjected to bioinformatic analysis (PubMed, GenBank, Entrez, Locuslink, OMIM, UniGene databases and search engines). Data about structure, expression, cellular localization, function and biological role of these genes have been considered in this analysis.
Finally, we have selected two candidate genes – Early Growth Response-1 Factor (EGR-1) and TAX-responsive enhancer element binding protein 107 (TAXREB107) – for the further work because of their more pronounced differential expression and their role in regulation of hematopoiesis described in the literature so far.

3.2. EGR-1 and TAXREB107: expression studies in patients

In experiments of identification of genes differentially expressed in GPI-positive and GPI-negative PNH cell lines we found EGR-1 and TAXREB107 to exhibit strong difference in expression level. Therefore, the objective of our further studies was to find relation between the observed overexpression and clonal expansion in PNH and investigate it in other hematological diseases.

3.2.1. Patients used for analysis

Ten patients with PNH followed up at Hannover Medical School were included in the study. The patients were aged between 35 and 60 years. All of them have been tested by flow cytometry for the expression of GPI-linked surface antigens. Five patients (S.N., A.P., T.C., A.S., W.E.) have more than 95% GPI-deficient granulocytes, two (K.G., J.S.) – 80-95%, and three (D.R., J.O., J.A.) – 50-80%. Four of them (A.P., T.C., A.S., E.W.) have been treated for

aplastic anemia (AA) before the diagnosis of PNH had been made. Cytological analysis of granulocytes revealed no changes in nuclear or cytoplasmic maturation. All patients were in good clinical condition without signs of infection, were not treated with G-CSF or GM-CSF. None of the patients had been transfused for at least 3 month prior to the time of study.
In addition, peripheral blood samples from two patients with autoimmune hemolytic anemia (AIHA), 2 with polycythemia vera, 1 with myelodysplastic syndrome after severe aplastic anemia (MDS after SAA), one with MDS transformed into AML after chemotherapy presenting with continuous dysplastic signs on cytologic analysis of bone marrow, eleven patients with other diseases such as thalassemia, CML, CMML, AML, ITP and ALL have been analyzed (Table 2). Healthy volunteers served as controls. One of healthy individuals was analyzed while having leucocytosis due to sinubronchial infection (WBC 15G/L).

3.2.2. Construction of EGR-1-specific and β-actin-specific templates for generation of radioactive probe for Northern blot analysis

For the Northern blot hybridization analysis we constructed gene specific probes (described in detail in *Methods*). Briefly, 769 bp fragment of EGR-1
cDNA was amplified by RT-PCR and cloned into pCR2.1 vector and sequenced (Figure 7). The fragment was cut out of plasmid by *EcoR* I and digested by *Ava* II in order to remove zinc finger coding region. Obtained 177 bp cDNA fragment (position 1008-1185 in GenBank sequence, accession # NM_001964) is specific for EGR-1 gene and served as template in generation of radioactive probe.
362 bp fragment of β-actin gene (position 467-829 in GenBank sequence, accession # BC016045) was also amplified by RT-PCR reaction, cloned into pCR2.1 vector, sequenced and excised by *EcoR* I enzyme. Radioactive probe for Northern blot analysis was generated using obtained fragment.

Patient	Diagnosis	Sex	Current therapy	GPI-deficiency (%)	blood counts		
					L (G/L)	Hb (g/dL)	T (G/L)
A.P.	PNH/SAA	f	steroids	> 95	6,2	7,6	49
S.N.	hemol. PNH	m	none	> 95	4,5	8,2	166
K.G.	hemol. PNH	m	none	80-95	5,7	13,3	217
D.R.	hemol. PNH	f	none	50-80	8,2	12,4	164
A.S.	PNH/SAA	m	Cyclosporin*	> 95	3,9	7,3	253
J.O.	hemol. PNH	m	Cyclosporin*	50-80	3,6	11,1	70
T.C.	PNH/SAA	m	Cyclosporin*	> 95	3,3	7,4	42
J.S.	PNH	f	none	80-95	3,7	10,7	129
W.E.	PNH/SAA	f	none	> 95	5,4	8,9	225
J.A.	PNH	f	none	60-80	3,6	13,1	93
D.W.	AIHA	m	MMF and steroids	-	5,7	9,9	77
P.W.	AIHA	f	steroids	-	18,3	13,5	677
C.W.	P.vera	f	Hydroxyurea	-	2,6	12,9	432
G.H.	P.vera	m	venous puncture	-	7,0	9,6	238
K.M.	SAA-MDS	m	SCF and G-CSF	-	3,8	8,2	7
H.W.	MDS-AML	f	intermittent chemotherapy	-	3,4	10,5	66
D.W.	ALL	m	G-CSF	-	23,5	9,6	57
A.K.	MPS	f	none	-	10,1	13,1	1009
J.F.	MPS	m	none	-	8,3	15,3	870
H.H.	MPS	m	transfusions	-	9,6	14,9	228
W.D.	CMML	m	Hydroxyurea	-	8,2	10,9	56
M.B.	OMF	m	Hydroxyurea	-	3,6	9,7	232
L.G.	ITP	f	Azathioprin and steroids	-	3,8	9,8	33
T.K.	CML	f	Hydroxyurea	-	7,8	11,5	230
G.K.	CLL	m	Chloramucil	-	33,8	10,8	99
V.D.	Talassemia	f	transfusions, splenectomy	-	17,6	8,9	983
I.D.	AML	m	none	-	5,7	9,9	115

Table 2: Characteristics of patients studied. * **These patients were treated with Cyclosporin not at the time of analysis.**

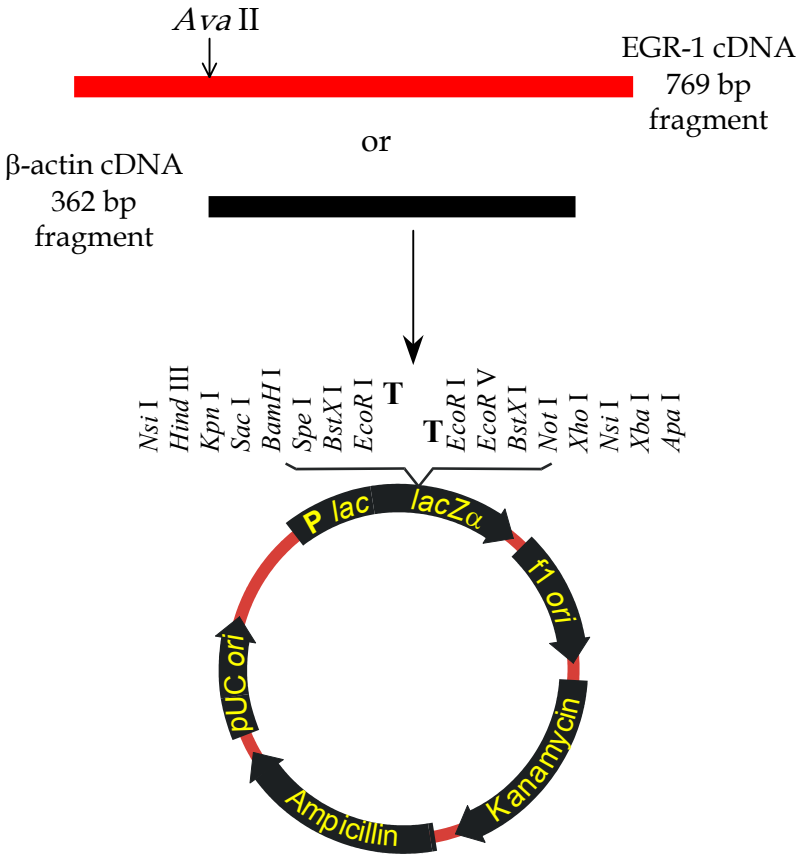

Figure 7: Construction of EGR-1-specific and β-actin-specific templates. Gene fragments were amplified by RT-PCR, cloned into pCR 2.1 vector and sequenced. For the preparation of radioactive probe inserts were cut out by *EcoR* I.

3.2.3. Expression of EGR-1 in PNH granulocytes

In order to study gene regulation in native PNH cells, we chose granulocytes because in most patients with primary hemolytic PNH the vast majority of granulocytes are GPI-deficient (>95%). In addition, granulocytes as myeloid cells are optimally representing the relation of GPI-deficient and normal progenitor cells in bone marrow of PNH-patient due to their short lifetime (Prince et al. 1995). Therefore, we confirmed differential expression of EGR-1 by Northern blot analysis using GPI-deficient granulocytes of PNH patients

in comparison to normal donors. Significantly upregulated EGR-1 gene expression (5 times more than level for normal donors) has been observed for all analyzed PNH patients (Figure 8).

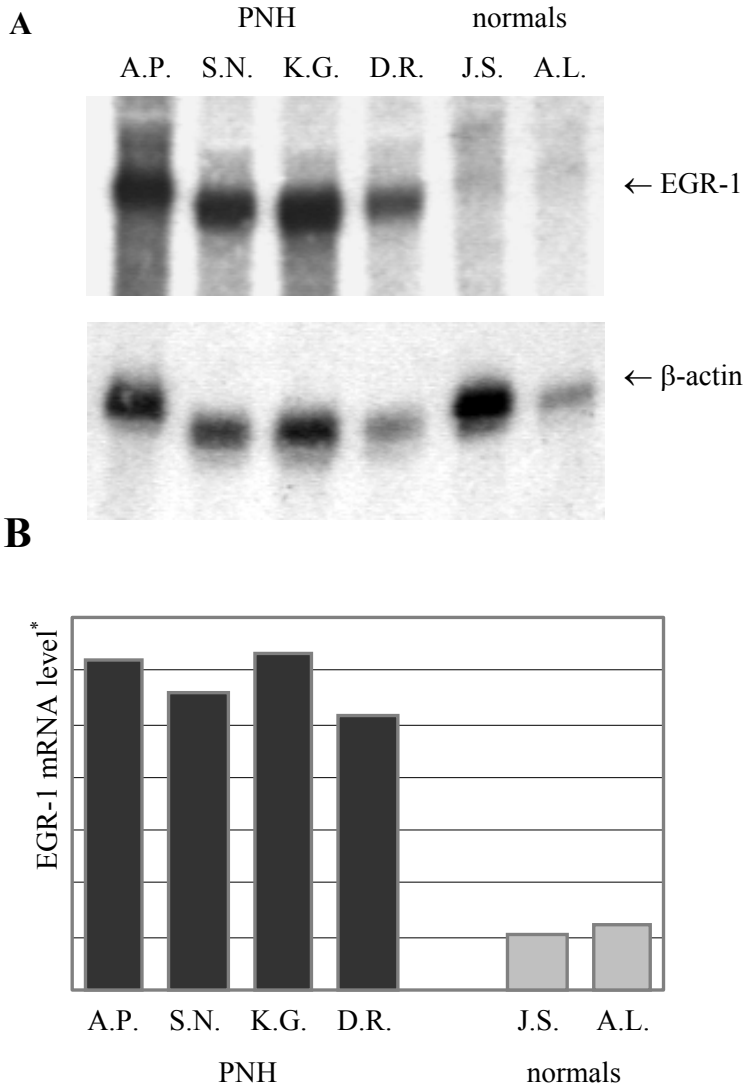

Figure 8: Expression of EGR-1 in granulocytes.* A: Representative Northern blot of total cellular RNA from GPI-deficient granulocytes of PNH patients in comparison to granulocytes of normal donors, probed with EGR-1 (upper panel) and actin (lower panel). B: Histogram showing densitometric analysis of representative Northern blot. For all analyzed PNH patients significant overexpression of EGR-1 has been observed.

* All bar diagrams are presenting mRNA levels quantified by PhosphorImager and normalized to β-actin.

3.2.4. Expression in granulocytes of patients with other hematological diseases

Expression of EGR-1 in granulocytes obtained from patients with other hematological diseases like MDS, SAA, AIHA was analyzed. Northern blot analysis revealed an overexpression of this gene only in some cases of myeloproliferative and myelodysplastic disorders. For normal donors and patients with hemolytic anemia and a positive Coombs test, the expression of EGR-1 was not significantly altered (Figure 9).

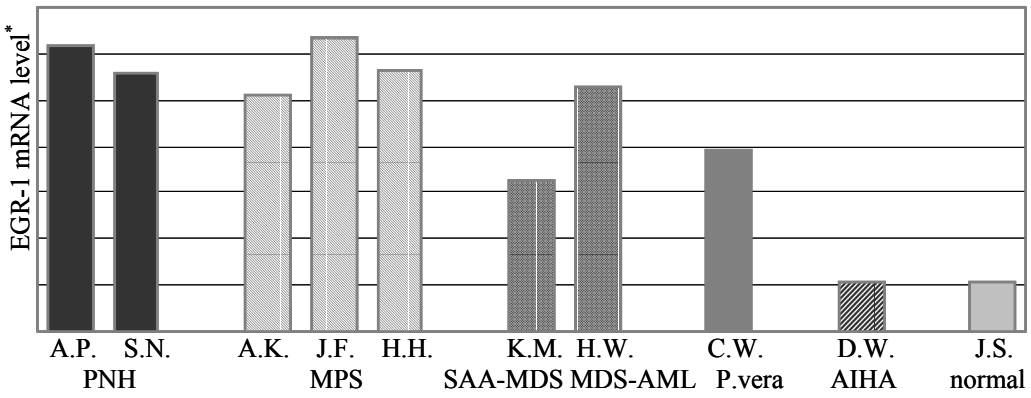

Figure 9: **Expression of EGR-1 in granulocytes of patients with other hematological diseases.* Analysis of expression in granulocytes obtained from patients with other hematological diseases revealed an overexpression of this gene also in some cases of myeloproliferative and myelodysplastic disorders.**

* All bar diagrams are presenting mRNA levels quantified by PhosphorImager and normalized to β-actin.

In addition, EGR-1 expression was tested in patients with thalassemia, CML, CMML, AML, ITP and ALL. In these patients no changes in EGR-1 expression level have been observed (Figure 10).

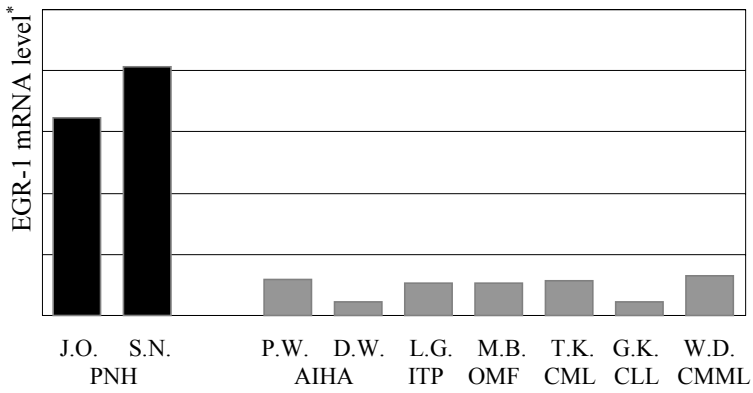

Figure 10: Expression of EGR-1 in granulocytes of hematological patients*. No overexpression of EGR-1 detected in cases of AIHA, ITP, OMB, CML, CLL, CMML.

3.2.5. Construction of TAXREB107-specific template for generation of radioactive probe for Northern blot analysis

For the Northern blot hybridization analysis we constructed TAXREB107 gene specific probe (described in detail in *Methods*). Briefly, 507 bp fragment of TAXREB107 cDNA was amplified by RT-PCR and cloned into pCR2.1 vector and sequenced (Figure 11). Than it was cut out of plasmid by *EcoR* I. Obtained fragment (position 51-558 in GenBank sequence, accession # X69391) served as template in generation of radioactive probe.

* All bar diagrams are presenting mRNA levels quantified by PhosphorImager and normalized to β-actin.

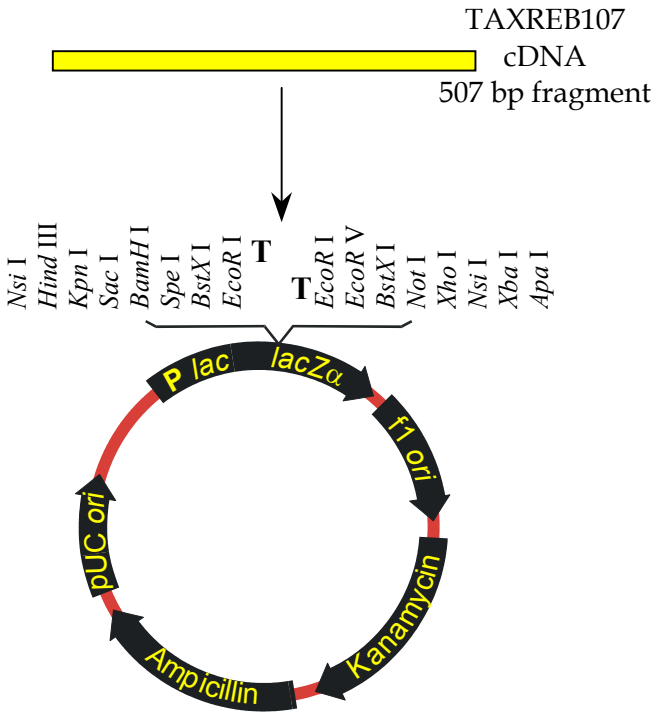

Figure 11: Construction of TAXREB107-spesific template. Gene fragment was amplified by RT-PCR, cloned into pCR 2.1 vector and sequenced. For the preparation of radioactive probe insert was cut out by *EcoR* I.

3.2.6. Expression of TAXREB107 in granulocytes of PNH patients and patients with related hematological diseases

Analysis of TAXREB107 gene expression in granulocytes of PNH patients revealed clear overexpression for three out of seven cases. All of these patients have been treated for aplastic anemia before the diagnosis of PNH. In at least one of these three patients (T.C.) signs of dyserythropoiesis not sufficient to diagnose MDS have been detected at the analysis of bone marrow smear. We observed comparable overexpression level for patients with MPS. In addition, overexpression of TAXREB107 has been found in a patient who

developed MDS after SAA without cytogenetic abnormalities and in another patient with AML after MDS whose bone marrow analysis revealed continuous dysplastic granulo- and erythropoiesis after regeneration from chemotherapy (Figure 12).

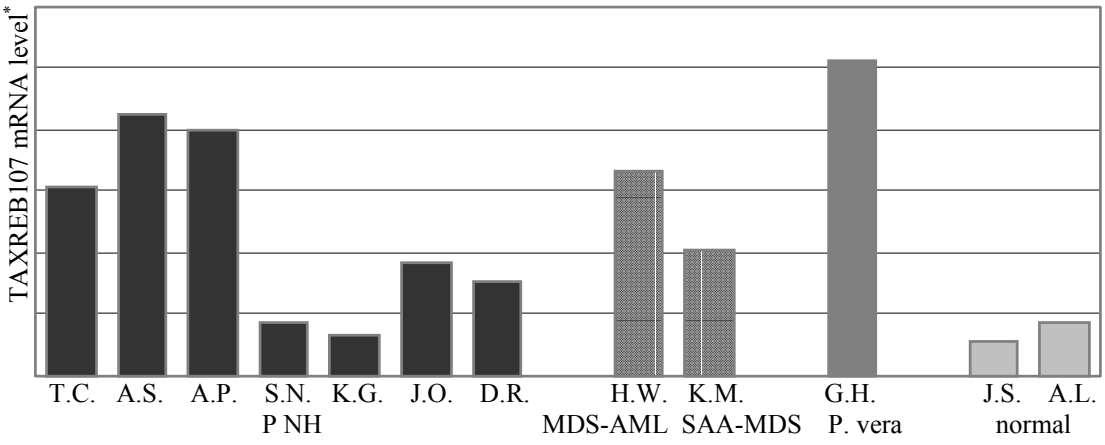

Figure 12: **Expression of TAXREB107 in granulocytes.* For three out of seven PNH patients clear overexpression of TAXREB107 has been observed. Comparable overexpression is detected in some cases of myeloproliferative and myelodysplastic diseases.**

3.3. Overexpression of Egr-1 and altered cytokine level

In order to exclude that the observed differences are secondary to external factors such as cytokines further investigations have been included. It has been demonstrated in PNH patients that plasma concentrations of endogenous hematopoiesis-regulatory cytokines (such as G-CSF) are increased in comparison to normal donors (Nakakuma et al. 1997). Moreover,

* All bar diagrams are presenting mRNA levels quantified by PhosphorImager and normalized to β-actin.

the cytokine profiles of PNH patients are quite similar to those of patients with aplastic anemia (AA) and myelodysplastic syndrome (MDS). As shown previously, EGR-1 is rapidly and transiently expressed in response to G-CSF and GM-CSF (Figure 13A). In order to exclude G-CSF as an external factor leading to a differential expression of EGR-1 in granulocytes of PNH patients, we tested its expression by Northern blot analysis in a patient receiving high doses of G-CSF (2×5µg/kg) for mobilization of CD34+ cells. Application of exogenous G-CSF did not lead to an overexpression of EGR-1 in the patients' granulocytes (Figure 13B).

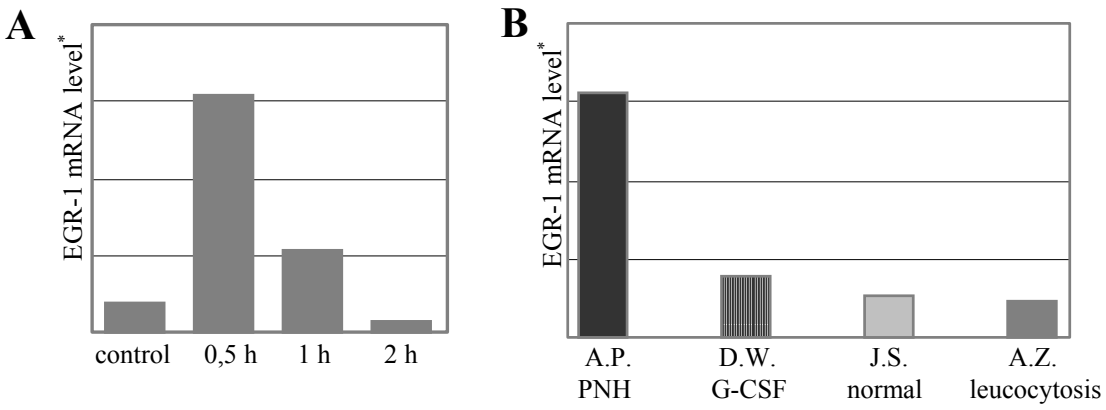

Figure 13: Overexpression of EGR-1 is not a result of altered levels of plasma cytokines.* A: In order to address external influences such as cytokines leading to a differential expression of EGR-1 in granulocytes, we analyzed the in *vitro* stimulation of granulocytes with G-CSF resulting in a short-term up-regulation of EGR-1. B: In contrast to *in vitro* stimulation, granulocytes obtained from patient, receiving high doses of G-CSF for hematopoietic stem cell mobilization, did not exhibit any increased EGR-1 expression. In addition, normal donor (A.Z.) with leucocytosis due to sinubronchial infection (15 G/L) demonstrated also normal revel of EGR-1 expression, comparable with value for that donor when healthy.

* All bar diagrams are presenting mRNA levels quantified by PhosphorImager and normalized to β-actin.

In order to exclude additional external factors present in the plasma of PNH patients, we tested EGR-1 expression in granulocytes from normal donor after incubation in patient's plasma. This experiment has been performed for different combinations of PNH patients and normal donors. Granulocytes from a normal donor have been incubated for 3 different periods of time in plasma of PNH patients compared to normal donors.

As shown on Figure 14 no significant change in EGR-1 mRNA level was observed in comparison to the control. In addition, the level of EGR-1 in granulocytes obtained from normal donors with reactive leukocytosis due to sinubronchial infection was not altered (Figure 13B, A.Z.). Although it can not be completely excluded, our results favour the explanation that EGR-1 overexpression in PNH is rather due to intrinsic cellular factors and not simply dependent from altered levels of plasma cytokines.

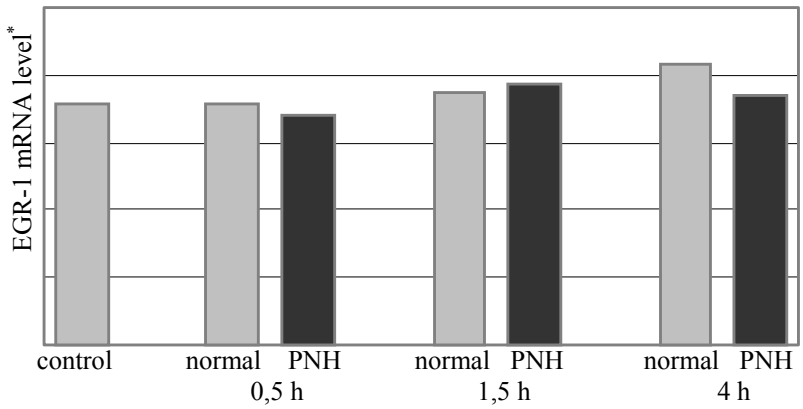

Figure 14: **Incubation of normal granulocytes in plasma of PNH patients. Granulocytes obtained from normal donor (J.S.) were incubated in plasma of PNH patient (A.P.) and other normal donor (A.L.) for 3 different time periods. No considerable changes in EGR-1 mRNA level* were observed. One representative out of three different experiments is given.**

* All bar diagrams are presenting mRNA levels quantified by PhosphorImager and normalized to β-actin.

3.4. EGR-1: analysis of up-stream transcriptional factors

Early Growth Responce-1 factor (EGR-1) is a transcriptional factor regulating expression of more than 30 genes, including growth factors and cytokines. EGR-1 is rapidly and transiently induced by growth factors and differential signals and is functionally involved in cell proliferation and differentiation. EGR-1 expression can be induced via mitogen activated protein (MAP) kinases. Several parallel MAP kinase signal transduction pathways have been defined in mammalian cells. These pathways include the extracellular signal regulated kinases (ERK), c-Jun N-terminal kinases (JNK, also known as SAPK1) and p38 MAP kinases (SAPK2) (Rolli et al. 1999). In addition, EGR-1 expression can be regulated via JAK-STAT pathway (Tian et al. 1996) despite the STAT5-binding site in direct proximity of *EGR-1* gene promoter is not reported. Figure 15 summarize the signal transduction pathway regulating expression of EGR-1.

Thus, we hypothesized that overexpression of EGR-1 might be a result of up-regulation of up-stream transcriptional factors. For this reason we investigated the expression of serum response factor (SRF), STAT5a and STAT5b as transcription factors regulating EGR-1.

We compared expression level of SRF, STAT5a and STAT5b for PNH patients with detected overexpression of EGR-1 and normal donors by Northern blot hybridization analysis.

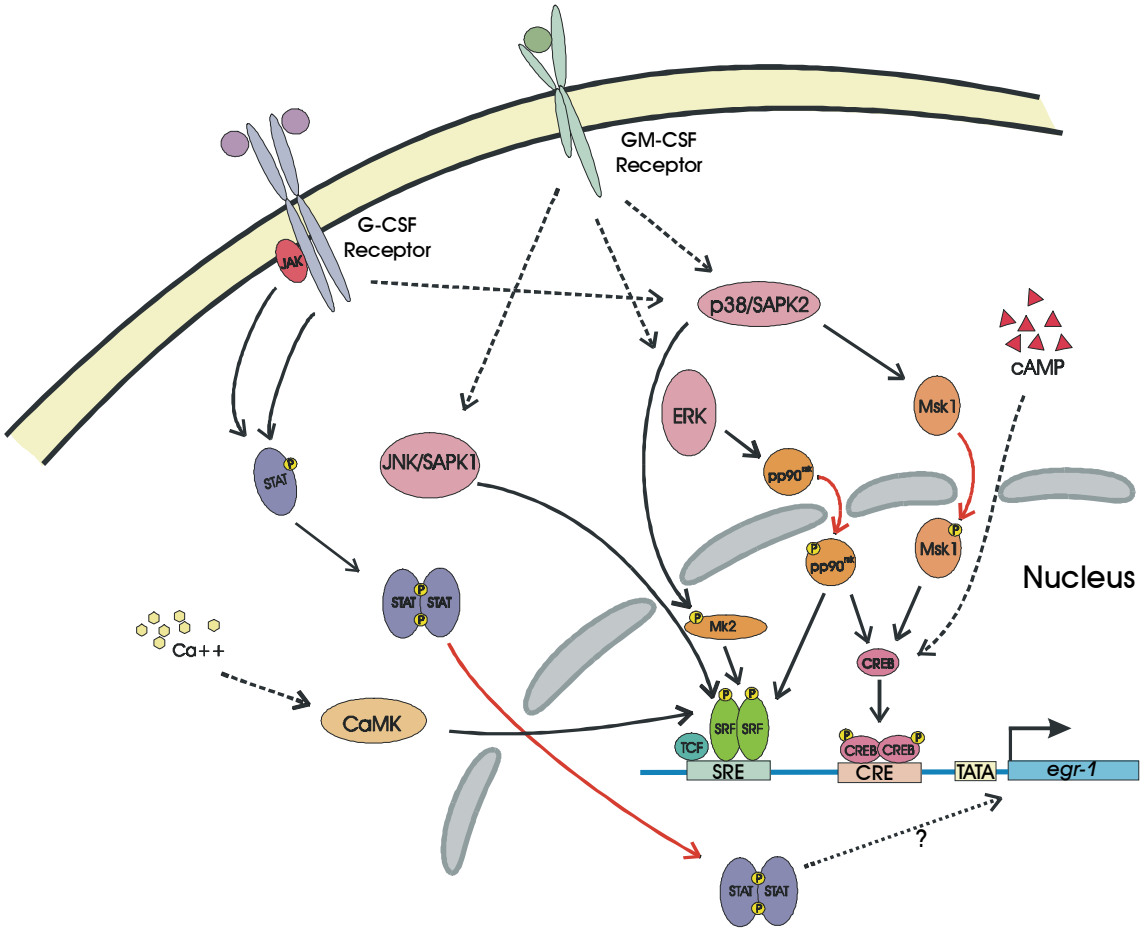

Figure 15: Signal transduction pathways inducing expression of EGR-1 gene. (according to (Tian et al. 1996; Watson et al. 1997; Khachigian and Collins 1998; Smithgall 1998; Rolli et al. 1999; Coffer et al. 2000; Mora-Garcia and Sakamoto 2000)).

3.4.1. Gene-specific templates for up-stream transcriptional factors used in Northern blot analysis

In order to study expression of SRF, STAT5a and STAT5b transcription factors in PNH patients exhibited overexpression of EGR-1 we chose Northern blot hybridization analysis. Cloned fragments of coding regions for these genes were obtained from resources of RZPD as bacteria stocks. Every plasmid was sequenced to confirm gene specificity of insert. Inserts were cut out by

digestion with restriction enzymes (Figure 16), purified and used as template for the radioactive probe synthesis.

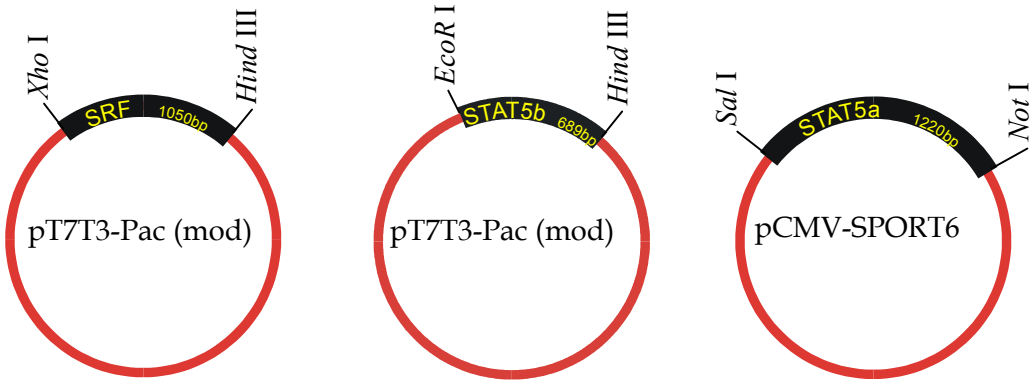

Figure 16: Preparation of SRF, STAT5a and STAT5b specific templates for generation of radioactive probes. Cloned fragments of genes were obtained from RZPD and excised by restriction enzymes as indicated.

3.4.2. Analysis of expression of SRF, STAT5a and STAT5b as regulators of EGR-1 transcription

In order to elucidate molecular bases of EGR-1 overexpression in PNH we compared expression level of SRF, STAT5a and STAT5b in granulocytes of PNH patients and normal donor by Northern blot hybridization analysis. No significant difference in expression was observed for any of them. Representative Northern blot probed for STAT5b is shown on the Figure 17. Therefore, we conclude that EGR-1 overexpression in PNH is not a consequence of possible overexpression of up-stream transcriptional factors.

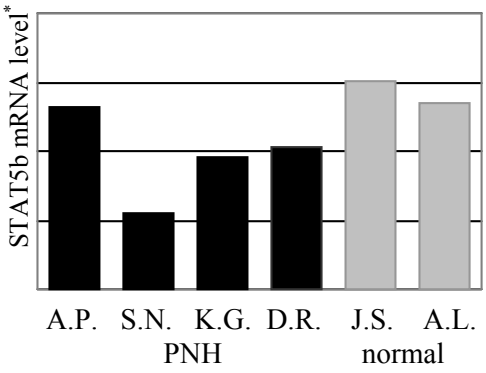

Figure 17: **Analysis of up-stream transcriptional factors for EGR-1*. Shown representative Northern blot probed for STAT5b. No change in expression level of STAT5b as well as STAT5a and SRF detected.**

3.5. Analysis of EGR-1 chromosomal organization, gene and transcript structures

We have shown that Early Growth Responce-1 factor (EGR-1) is overexpressed in GPI-deficient granulocytes of all PNH patients studied so far.

EGR-1 belongs to the class of early response genes and can be rapidly and transiently expressed in response to different stimuli. We demonstrated that overexpression of EGR-1 in PNH is not a result of altered plasma cytokine level (*Results, 3.3*). In addition, we did not observed any changes in expression level of EGR-1 up-stream trancriptional factors (factors regulating EGR-1 transcription) for PNH patients in comparison to normals (*Results, 3.4.*).

* All bar diagrams are presenting mRNA levels quantified by PhosphorImager and normalized to β-actin.

Therefore, analysis of EGR-1 chromosomal organization (FISH experiments), EGR-1 promoter structure and structure of the gene coding region was performed in order to analyze EGR-1 overexpression in PNH.

3.5.1. EGR-1 promoter studies

In order to analyze the primary structure of EGR-1 promoter (Figure 18) we constructed plasmids harboring the EGR-1 promoter fragment spanning nucleotide –674 to +12. We performed 2 parallel PCR reaction for every PNH patient and normal donor and sequenced at least 3 different plasmids for every cloned PCR fragment. DNA sequencing analysis of both DNA strands was performed as described.

Obtained sequences were analyzed using GeneRunner software and searched against GenBank, dbEST and SwissProt databases through the BLAST server.

We detected that EGR-1 promotor sequence for all PNH patients studied is identical to the corresponding region of normal donors and to the published complete nucleotide sequence.

3.5.2. EGR-1 coding region studies

The human *EGR-1* gene is composed of two exons and one intron, spanning approximately 3,6 kb (Sakamoto et al. 1991). EGR-1 belongs to the EGR family of zinc-finger transcriptional factors. EGR-1 protein is 544 aa long (accession # P18146) and has three zinc-finger C_2H_2-type domains and one transactivation domain (Figure 19).

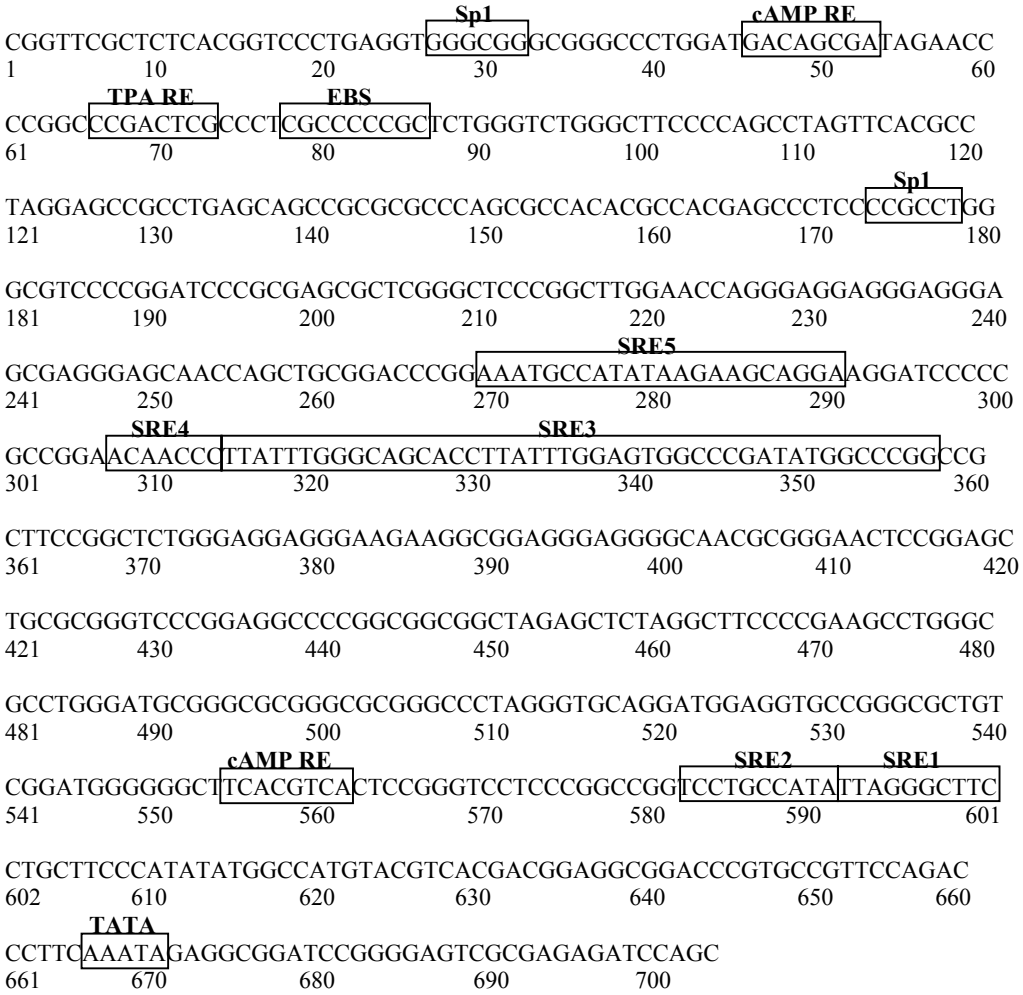

Figure 18: Complete nucleotide sequence of the human EGR-1 promoter. Regulatory sequence elements are boxed (according to (Schwachtgen et al. 2000)). Sp1 = Sp1 binding site; cAMP RE = cyclic AMP response element; TPA PE = 12-O-Tetradecanoyl phorbol 13-acetate response element; EBS = EGR-1 binding site; SRE = serum response element; TATA = TATA box. (GeneBank accession number AJ245926).

It is important to note that the transactivation domain of *EGR-1* gene harbors six trinucleotide AGC repeats at two regions in nucleotide positions 454 to 471 and 505 to 522 ($AGC_{6/33/6}$) according to GenBank NM_001964 (Gashler and

Sukhatme 1995). The transactivation domain activates transcription 100-fold. Mutations in the transactivation domain may abrogate the EGR-1 transcriptional activity.

We analyzed the primary structure of *EGR-1* gene coding region. For that reason several fragments overlapping the ORF were amplified by RT-PCR and PCR from genomic DNA and sequenced (described in detail in *Methods*).

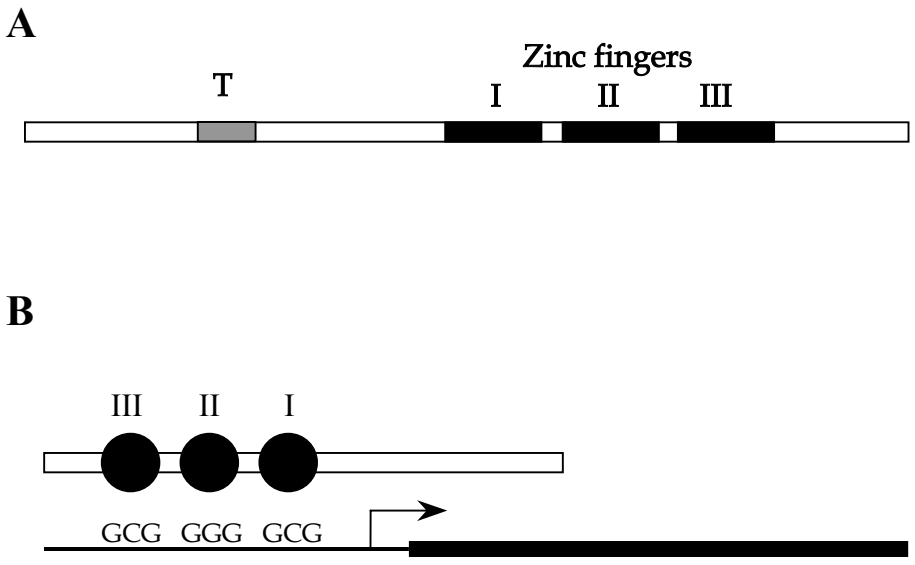

Figure 19: EGR-1 as a transcriptional factor. A: Domains of EGR-1 protein are shown. Three zinc-fingers DNA-binding domains are labeled accordingly (shown in black). The transactivation domain is shown in gray. B: EGR-1 is shown binding to the canonical EGR response element (RE) upstream of hypothetical target gene. Each zinc-finger (black circle) binds to a three-nucleotide site in an antiparallel configuration with zinc-finger I binding to the 3′-most nucleotide triplet and zinc-finger III binds to the 5′-most nucleotide triplet.

We amplified and sequenced the EGR-1 coding region from both genomic DNA and RNA obtained from four PNH patients with EGR-1 overexpression. We detected no significant changes or earlier described mutations within the primary structure of the coding region compared to EGR-1 GeneBank sequences XM_004063 for mRNA and NM_001964 for genomic DNA. However, for every patient among with normal clones we found some clones (approximately 1/3 out of all analyzed clones containing the corresponding region) with single nucleotide changes (shown in the Table 3).

Patient	RCR fragment	clone	comparison to GeneBank XM_004063
A.P.	sense+5038r cDNA	1b	position 1559 T→C position 1561 T→C
W.E.	sense+5038r genomic	4b	position 1354 A→G (zinc-finger domain I)
J.S.	sense+5038r cDNA	5b	position 1583 T→C
J.A.	sense+5038r genomic	8b	position 1522 A→G (zinc-finger domain III)

Table 3: Point mutations in EGR-1 coding region found in PNH patients with EGR-1 overexpression.

All detected molecular changes are located within the part of the coding region encoding for zinc fingers. In addition, two of detected point mutation (patients W.E. and J.A.) are located directly within zinc fingers I and III respectively. The second histidine in the consensus structure of C_2H_2 zinc-finger (Tyr/Phe–X–Cys–X_{2-4}–Cys–X_3–Phe/Tyr–X_5–Leu–X_2–His–X_{3-4}–His) is affected in both cases and exchanged to arginine. Such molecular change might lead to a significant alteration of EGR-1 protein function as a transcriptional factor due to alteration of zinc-finger affinity to DNA.

However, this assumption has to be verified on a larger series of patients and on experiments providing inside into the effects on cellular biology due to mutated EGR-1.

3.5.3. FISH analysis

In order to study chromosomal organization of EGR-1 and detect possible gene amplification or loss of heterozygosity we performed FISH analysis with molecular probe to EGR-1 (5q31) loci for granulocytes obtained from blood of normal controls and PNH patients. We modified the standard technique by choosing granulocytes instead of whole blood for analysis because of their up to 100% GPI-deficiency in PNH. Thus, it allowed us to directly compare the normal cells and PNH-clone.

For the FISH analysis we used probe LSI 5q EGR1 SO/ D5S23 SG obtained from Vysis, Inc. This FISH probe is generally used to determine the presence, amplification or absence of EGR-1 loci (5q31 band) and appears as an orange-red signal. As an internal control locus D5S23 (15p15.2 band) was used which appears as a green signal. The hybridized probe fluoresces with moderate to bright intensity both in interphase nuclei and on metaphase chromosomes. In the nuclei of normal cells, the probe generally appears as four distinct signals (two red and two green). Occasionally, the probe may appear as five or six signals, depending upon the condensation of the DNA and the relative distances between chromatids. The signals may also appear diffuse or split. In normal metaphase spreads, the probe may appear as two to four signals on each chromosome 5.

We analyzed granulocytes of three PNH patients with observed overexpression of EGR-1 and compared them to normal granulocytes. The results of fluorescent microscopy are presented on the Figure 20. Two red (EGR-1 loci, 5q31) and two green (control, 15p15.2) signals were clearly

detectable in the vast majority of nuclei of either normal or PNH granulocytes. In addition, for every sample we counted signals of 200 nuclei. Counting data are presented in the Table 4. Table 4 and Figure 20 indicate that PNH and normal samples were visually undistinguishable from each other and 99% of cells exhibit counts characteristic for normal cells. Therefore, it can be concluded that EGR-1 overexpression in granulocytes of PNH patients is not a consequence of any chromosomal aberration within the EGR-1 loci such as gene amplification.

Sample	# cells, 0 dots red / green	# cells, 1 dot red / green	# cells, 2 dots red / green	# cells, 3 dots red / green	# cells, >3 dots red / green
Normal (**A**)	0 / 0	4 / 3	195 / 196	1 / 0	0 / 1
PNH (**B**)	1 / 0	5 / 4	194 / 195	0 / 1	0 / 0
PNH (**C**)	0 / 0	2 / 1	196 / 194	2 / 4	1 / 1
PNH (**D**)	0 / 0	1 / 0	199 / 198	0 / 2	0 / 0

Table 4: FISH analysis of granulocytes of PNH patients and normal control. Counting data for 200 nuclei are presented. Healthy control and PNH patients are indicated A, B, C and D corresponding to images on the Figure 20.

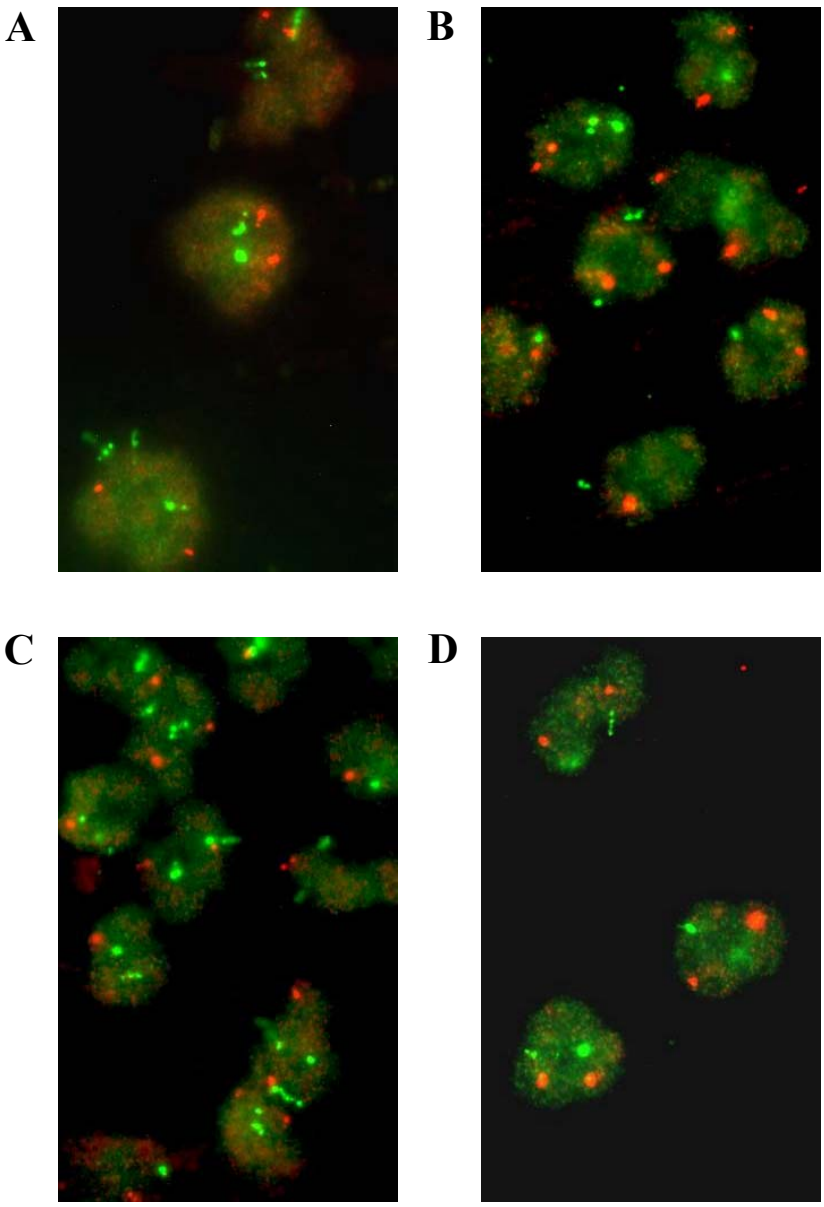

Figure 20: FISH molecular analysis of normal and PNH granulocytes. Granulocytes were isolated from fresh whole blood, fixed by methanol/acetic acid and hybridized with LSI 5q EGR-1 SO / D5S23 SG dual color DNA probe. Red signals are corresponding to the band 5q31, EGR-1 loci; green signals – 5p15.2, locus D5S23. Panel A: normal donor (J.S.), panels B, C, D: PNH patients (J.A., A.P. and W.E. respectively).

4. Discussion

4.1. Clonal expansion in PNH: a consequence of immune selection or of additional gene alterations?

The pathophysiology leading to the expansion of GPI-deficient clones within the bone marrow of PNH or PNH/aplasia patients is still a matter of controversial discussions (Dunn et al. 1999). Especially, the frequent appearance of expanded GPI-deficient bone marrow cells in aplastic anemia patients has led to the hypothesis that GPI-deficient cells as a result of clonal expansion would escape the suppressive action of the immune system (Dunn et al. 1999). This is further supported by the observation that in some PNH patients more than one GPI-deficient clone is present (Bessler et al. 1994; Nafa et al. 1995). However, there are no investigations reported about the size of each clone contributing to the GPI-deficient cell fraction so far. If all these clones would exhibit an equal size, an absolute growth advantage of one clone dominating the bone marrow would be unlikely. However, if there would be one dominating and several minute size clones as described in normals (Araten et al. 1999) this would not rule out such an absolute growth advantage. GPI-deficient cells exhibit the same susceptibility to the cytotoxic action of allogeneic T-cells as normal cells do (Karadimitris et al. 2000). Thus, a direct recognition of GPI-linked proteins or GPI-anchor molecules presented at the surface of hematopoietic stem cells by immunological effector cells needs to be anticipated (Porcelli and Modlin 1999). On the other hand, cells of virtually all tissues express GPI-anchors. Therefore, it would be necessary to anticipate that the cytotoxic reaction could distinguish between hematopoietic and non-hematopoietic cells because otherwise the resulting tissue damage would not be compatible with life. In addition, such an organ destruction is not present in the list of clinical features of PNH or AA. It has been proposed

that the autoimmune attack restricted to HSC could be associated with an expansion of cytotoxic T/NK cells recognizing GPI-anchor structures in the context of CD1d molecules (Schofield et al. 1999; Taniguchi and Nakayama). Such clones have been found in other autoimmune diseases, but not in PNH or AA patients so far. An alternative explanation could be that GPI-linked surface proteins such as ULBP's (cytomegalovirus glycoprotein UL16 binding proteins) (Cosman et al. 2001) might directly interfere with activating receptors (NKG2D) on NK cells (Pende et al. 2001). The expression pattern of these molecules is unknown to the date. Should they turn out to be HSC-specific, the restriction of the autoimmune reaction to HSC could be possibly explained.

As an alternative to the immune escape it was suggested that GPI-deficiency itself leads to an increased resistance to apoptosis (Brodsky et al. 1997). An increased rate of apoptosis has been observed in patients with aplastic anemia or myelodysplastic syndrome (Heaney and Golde 1999; Killick et al. 2000; Novitzky et al. 2000). If GPI-deficient cells would exhibit such a resistance due to only the absence of GPI-linked surface molecules, clonal dominance of the GPI-deficient clone would result in the context of AA or MDS. However, this observation could not be reproduced by other groups (Ware et al. 1998; Bastisch et al. 2000). Therefore, it has to be anticipated that resistance to apoptosis observed in GPI-deficient cells of PNH patients would be a result of changes distinct from the deficient surface expression of GPI-linked proteins.

GPI-deficient cells present in PNH exhibit an increased genetic instability, which is also observed in related diseases with defined genetic aberrations such as MDS (Hattori et al. 1997; Purow et al. 1999).

Therefore, we postulated that there are such additional alterations affecting genes regulating cellular proliferation and altered growth of GPI-deficient cells and, thus, responsible for clonal expansion in PNH.

4.2. Differential gene expression in PNH

In order to elucidate the molecular basis of clonal expansion in PNH we identified genes differentially expressed in normal and GPI-deficient cells. To enhance the productivity of a such analysis we have employed combination of two divergent approaches. cDNA Atlas array hybridization let us to investigate expression of genes already reported to play a role in human hematopoiesis and belonged to different classes – transcription regulators, cytokines and cell surface markers. RNA fingerprinting is based on PCR-mediated differential screening and has a significant advantage that it can be performed starting with only an amount of RNA as small as 0,1 μg. Moreover, due to an amplification step it has a different sensitivity and therefore allowed us to isolate diverse set of genes comparing to the Atlas cDNA array hybridization method.

We could demonstrate that GPI-positive and GPI-negative cells of the same PNH patient have different gene expression profiles. This could be explained by the observation of increased genetic instability ratio in GPI-deficient cells of PNH-patients reported recently (Hattori et al. 1997; Purow et al. 1999). Thus, a genetic instability would be the basis for molecular changes leading to an altered gene expression pattern.

We have chosen 2 candidates out of 26 differentially expressed genes for further study according to their pronounced variation in expression level and their previously described role in regulation of hematopoiesis.

4.3. TAX-responsive enhancer element binding protein 107 (TAXREB107)

TAXREB107 (TAX-responsive enhancer element binding protein 107) has been described as a protein mediating DNA binding for HTLV I trans-

activator of transcription (*tax*) for its own viral promoter (Sodroski et al. 1984), and also for some promoters of cellular genes such as IL-2, IL-2Rα, GM-CSF and *c-fos* (Nagata et al. 1989). TAXREB107 has a leucine zipper motif and binds specifically to the C-domain of *tax*-responsive enhancer element, which has some similarity with the cAMP response element (CRE). However, the function of TAXREB107 in uninfected cells has not yet been completely clarified. Nevertheless, it appears to be upregulated upon the action of erythropoetin (Li et al. 1999).

Dyserythropoiesis has been described in a series of PNH (Iwanaga et al. 1998; Dunn et al. 1999). In the patients analyzed here, maturation disturbances have been present in those patients providing an increased level of TAXREB107 transcription. These results need to be verified in a larger series of PNH or AA patients. However, TAXREB107 could be a candidate gene indicating additional changes such as an increased cycling stress within erythroid progenitors.

4.4. Early Growth Response-1 Factor (EGR-1)

Early growth response gene EGR-1 (Varnum et al. 1989) (also known as Krox 24, Tis 8, zif-268, G0S30 and NFGI-A) is a ubiquitously expressed zinc finger transcription factor (59kDa) (Cao et al. 1990) which can act to either positively or negatively regulate gene transcription (Gashler et al. 1993; Lin and Leonard 1997). Its ambivalent effect on regulation of hematopoiesis has been described (Varnum et al. 1989; Nguyen et al. 1993; Dinkel et al. 1998). EGR-1 was first identified as putative G_0/G_1 switch regulatory gene in human blood lymphocyte cultures and named G0S30 (Forsdyke 1985). It has been demonstrated to be a critical upstream mediator of proliferation (Perez-Castillo et al. 1993), differentiation (Nguyen et al. 1993; Dinkel et al. 1998), and apoptosis (Muthukkumar et al. 1997; Nair et al. 1997). In conditions of stress,

EGR-1 acts as an antiapoptotic gene and counteracts p53-dependent apoptosis (de Belle et al. 1999). Moreover, physical interaction between p53 and EGR-1 proteins has been recently reported (Liu et al. 2001). In addition, the EGR-1 gene product has decreased expression and activity during cellular senescence (Meyyappan et al. 1999).

In our investigations, we have found EGR-1 to be strongly up-regulated in all studied cases of PNH and in some cases of myeloproliferative and myelodysplastic diseases. MDS and MPS are generally assumed as clonal diseases driven by molecular changes within their activation gene program (Killick et al. 2000). Furthermore, there are certain similarities and some overlap between PNH and MDS (Young and Maciejewski 2000). We assume that differential regulation of EGR-1 as a gene, playing a role in the regulation of differentiation, proliferation, apoptosis and regulation of the cell cycle, could be associated with expansion of GPI-deficient clones in PNH. In our experiments we have also shown that overexression of EGR-1 in PNH is not due to extrinsic factors such as increased level of cytokines but is rather caused by intracellular changes.

To further investigate the molecular basis behind upregulated *EGR-1* gene expression we analyzed *EGR-1* chromosomal and gene organization. We detected no mutations whithin the promoter region of *EGR-1* gene and no molecular aberrations in ERG-1 loci according to results of FISH analysis. However, when sequencing the EGR-1 coding region, we detected some clones with point mutations despite the majority of analyzed clones had the sequence identical to normal. Interestingly, all detected mutations are located in the region encoding for zinc-finger domains. It is well known that EGR-1 is a zinc-finger transcriptional factor. Therefore, mutations in such a region might strongly influence the protein function.

However, it cannot be concluded strait forward that EGR-1 is connected directly with the growth advantage of the GPI-deficient hematopoietic cells. Moreover, there is even no definite information, which expression level of

EGR-1 would be sufficient for clonal expansion and which level would be deleterious for the function of hematopoietic stem or progenitor cells (Krishnaraju et al. 1998). The statement of a genetic basis such as EGR-1 overexpression in clonal dominance is further complicated by the observation that the proportion of GPI-deficient cells in PNH can be stable over a prolonged period of time (Schrezenmeier et al. 2000; Maciejewski et al. 2001).

It was recently reported that EGR-1 mRNA level is expressed at a significantly higher level in cancer cells than in normal tissue (Eid et al. 1998). In addition, the structure of the *EGR-1* gene implies that it may particularly be subject to microsatellite instability because of nucleotide repeats at three exon regions (Gashler and Sukhatme 1995). It appears to be very interesting taking into account our findings of point mutations in *EGR-1* gene region encoding C_2H_2 zinc-fingers domains. For two patients the second histidine coordinating zinc ion in zinc-fingers structure is replaced by arginine. Such molecular alterations would shift the affinity of zinc-finger to DNA and might lead to significant changes in EGR-1 transcriptional factor function. This could even be responsible for the revealed overexpression of EGR-1 in PNH due to the presence of an EGR-1 binding site in promoter region of *EGR-1* gene. In addition, the functional role of EGR-1 within the cell favors the hypothesis that any changes in *EGR-1* gene expression or activity might be a primary alteration that occurred in PNH cells.

It has recently been reported that EGR-1 directs murine myelopoiesis to the monocytic lineage on expense of self-renewal of pluripotent hematopoietic stem cells (Krishnaraju et al. 1998; Krishnaraju et al. 2001). This is of particular interest because of relative monocytosis indicates an ongoing clonal evolution such as PNH or MDS in the course of aplastic anemia (Nissen et al. 2000). These observations support our hypothesis that differential regulation of EGR-1 in hematopoietic stem cells could provide conditions for clonal expansion such as in PNH. However, the recently described increased genetic

instability (Hattori et al. 1997) may possibly result in more than one alteration of gene expression which could be responsible for clonal expansion in PNH.

4.5. Molecular changes in PNH

In this work, we found that molecular changes additional to *PIG-A* gene mutations are presented in PNH. Moreover, these gene expression alterations can be detected in hematological diseases with a related pathogenesis such as aplastic anemia, myelodysplastic and myeloproliferative syndromes. In addition, the research group of T. Kinoshita recently reported mutation in a HMGI-C/HMGA2 transcriptional factor (Inoue et al. 2001), a second genetic mutation confined to GPI-deficient cells in PNH, which in fact favors the hypothesis that clonal expansion in PNH is driven by additional molecular alterations.

Since underlying genetic defects leading to the upregulation of EGR-1 and TAXREB107 have not yet been uncovered, it might also be possible that the observed expression changes result from genetic alterations located within direct proximity of both genes. However, our results imply that the observed altered regulation is not a result of extracellular factors but merely of an altered genetic program possibly due to a clonal transformation process.

Taken together, these results indicate, that in clonal cells from PNH patients molecular events distinct from regulation by the immune system or resistance to apoptosis are presented. The observed differential gene expression or mutations whithin functional domains of genes regulating cellular growth could be responsible for clonal expansion of GPI-deficient cells in patients with PNH. Further studies will be required to confirm genetic alteration at a molecular level. This could be the basis for establishing more sensitive diagnostic tests in order to further understand and distinguish the pathophysiology of clonal disease such as PNH.

5. List of abbreviations

aa	amino acids
Ag	antigen
APS	ammonium persulfate
ATCC	American Type Culture Collection
BM	bone marrow
bp	base pair
BSA	bovine serum albumin
CD	cluster of differentiation
cDNA	complementary DNA
cpm	Counts per minute
DEPC-water	diethyl pyrocarbonate treated-water
DMEM	Dulbecco´s modified Eagle´s medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
ER	Endoplasmic reticulum
EGR-1	Early growth response –1 factor
FCS	fetal calf serum
FISH	fluorescence *in situ* hybridization
FITC	fluorescein isothiocyanate
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPI	glycosylphosphatidylinositol
h	hour
HSC	hematopoietic stem cell
IL	interleukin
IPTG	isopropyl-β-D-thiogalactoside
kb	kilobase pairs

MACS	magnetic cell separator
min	minute
M-MLV	Molony murine leukemia virus
mRNA	messenger ribonucleic acid
Neo^R	neomycin resistance
OD	optical density
ORF	open reading frame
PB	peripheral blood
PBS	phosphate buffered saline solution
PCR	polymerase chain reaction
PE	phycoerythrin
PIG-A	phospatidyinositol glycan complementation class A
PNH	paroxysmal nocturnal hemoglobinuria
RNA	ribonucleic acid
rpm	rotations per minute
RT	room temperature
RT-PCR	reverse transcriptase-polymerase chain reaction
RZPD	Resource Centre of the German Human Genome Project at the Max-Planck-Institute for Molecular Genetics
SB	sample buffer
SDS	sodium dodecyl sulfate
sec	second
SRF	serum response factor
STAT	signal transducer and activator of transcription
TAXREB 107	TAX-responsive enhancer element binding protein 107
UV	ultraviolet
v/v	volume/volume
w/v	weight/volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

6. List of producers

Amersham Pharmacia Biotech, Braunschweig, Germany

Amgen, Thousand Oaks, California, USA

Applichem GmbH, Darmstadt, Germany

ATCC, American Type Culture Collection, Manassas, Virginia, USA

Baker Company, Inc., Sanford, Maine, USA

B. Braun, Melsungen, Germany

Beckmann, Muenchen, Germany

Becton Dickinson GmbH, Heidelberg, Germany

Bender and Hobein AG, Zurich, Switzerland

Biochrom KG, Berlin, Germany

Bio-Rad Laboratories GmbH, Muenchen, Germany

Calbiochem-Novabiochem GmbH, Schwalbach, Germany

Cambridge Technology, Inc., Watertown, Massachusetts, USA

CellGenix, Freiburg, Germany

Cinna Biotech Labs Inc., Houston, Texas, USA

Clontech GmbH, Heidelberg, Germany

Cytogen®, Lahmer, Germany

DAKO, Hamburg, Germany

Difco Laboratories, Detroit, USA

Eppendorf, Hamburg, Germany

Eurogentec, Seraing, Belgium

Falcon, New Jersey, USA

Gelaire, Meckenheim, Germany

Gibco BRL, Neu-Isenburg, Germany

Greiner GmbH, Frickenhausen, Germany

Hannover medical school (MHH), Hannover, Germany

Hawksley & Sons Limited, Sussex, UK

Heidolph, Schwabach, Germany

Heraeus, Hanau, Germany

H. Juergens & Co., Bremen, Germany

ICN Biomedicals GmbH, Eschwege, Germany

Immunex Corp, Seattle, Washington, USA

Immunotech GmbH, Hamburg, Germany

International Biotechnologies Inc, New Haven, Connecticut, USA

Invitrogen BV, Groningen, Netherlands

Knick, Berlin, Germany

Landgraf, Hannover, Germany

Leitz, Oberkochen, Germany

Life Technologies, Gaithersburg, Maryland, USA

LKB, Wallac, UK

Macherey-Nagel GmbH, Dueren, Germany

MBI Fermentas Molecular Biology GmbH, St. Leon-Rot, Germany

Merck KGaA, Darmstadt, Germany

Millipore GmbH, Eschborn, Germany

Miltenyi Biotec, Bergisch Gladbach, Germany

MWG-Biotech, Ebersberg, Germany

Nalge Nunc International, Roskilde, Denmark

New Brunswick Scientific Co. Inc., Edison, New Jersey, USA

New England BioLabs, Beverly, Massachusetts, USA

Nikon, Surrey, UK

PAA Laboratories GmbH, Coelbe, Germany

Pharmacia, Freiburg, Germany

PharMingen GmbH, Hamburg, Germany

Promega, Mannheim, Germany

Qiagen GmbH, Hilden, Germany

Roche Diagnostics GmbH, Mannheim, Germany

Savant, Farmingdale, New York, USA

Seromed, Berlin, Germany

Serotec, Oxford, UK

Serva, Heidelberg, Germany

Shimadzu Deutschland GmbH, Duisburg, Germany

Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany

Stratagene GmbH, Heidelberg, Germany

RZPD, Resource Center of the German Human Genome Project at the Max-Planck-Institute for Molecular Genetics, Berlin, Germany

Takara, Otsu, Japan

Tebu GmbH, Frankfurt/M., Germany

Vysis Inc., Downers Grove, IL, USA

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Acknowledgement

In conclusion I would like to say a few, but probably most important words of gratefulness to all those people, who shared their time and efforts during my doctoral thesis.

To **Prof. Dr. Reinhold E. Schmidt** for giving the possibility to accomplish this work in his department along with taking over to be the Korreferent;

To **PD Dr. Jörg Schubert**, my scientific supervisor, for his helpful suggestions, very constructive and sparkling discussions, without which this work would never been done, as well as for careful reading and commenting on this manuscript;

To **Prof. Dr. Walter H. Müller** for acquiring the role of Referent and his comfortable assistance with all the formal processing and particularly with the scheduling;

I also thank all my colleagues, members of the department of Clinical Immunology (Anja, Bettina, Britta, David, Dimitrios, Engelbert, Esther, Gabi, Gamal, Hendrik, Jörg, Julia, Juriaan, Karsten, Kerstin, Margot, Marion, Martin, Nelli, Olga, Roland, Sabine, Silke, Sonja, Tanja, Torsten), who were always friendly and have cared for a relaxed and funny climate in the lab, for emotional support.

Thanks to Dr. Doris Steinemann for a lot of help, for providing me with tips and tricks, for efficient and pleasant discussions.

Thanks to my friends who always listened to my problems, though some of them sometimes did not even know what I was talking about.

Special thanks to my parents for strong support in everything and to my husband for tolerance, encouragement and love.

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September 2000	14th European Immunology Meeting EFIS 2000, Poznan, Poland, September 23-27, 2000. Liakicheva A, Ziolek A, Schmidt RE and Schubert J. 2000. „Isolation of candidate genes responsible for clonal expansion in PNH." *Immunol. Lett*. 73 (2,3): 238.
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