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DISERTAČNÍ PRÁCE

Expression of adaptor protein PAG/Cbp in non-neoplastic and neoplastic lymphoid tissue

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Contents:

1.0 Introduction

1.1 General remarks

It was the recent progress in cellular biology that shed new light on the process of neoplastic transformation, lead to the development of new therapeutic regimens in oncology and prompted research into identification of other potential molecular target molecules for newly developed drugs. Promising therapeutic targets are molecules involved in cell signalling (1-9).

Despite enormous progress in molecular biology and pathology in recent years there are still considerable gaps in understanding signalling pathways and their mutual relationships. New molecules involved in oncogenesis have been identified and still new are being reported with high frequency making it difficult to keep up with the recent knowledge, particularly for routine surgical pathologists. However, it is more and more apparent that modern pathology will embrace morphology and molecular pathology whose cell communication or cell signalling deregulation is an integral part. Articles suggesting new physiological or pathological roles of the signalling molecules represent a considerable proportion of published studies in a number of medical journals.

Accordingly with this trend, I studied expression of the molecule known as PAG/Cbp in lymphoid tissue by use of routine immunohistochemical procedures. PAG is involved in regulation Src-family kinases (SFK) whose activation precedes downstream propagation of extracellular signals along diverse signalling pathways as explained later in the text.

Objectives of my study were to determine expression pattern of PAG/Cbp in lymphoid tissue, to identify possible specific associations with ML entity(ies), and to find out whether PAG detection could be useful in diagnostic haematopathology.

1.2 Cell signalling

1.2.1 Principles

Cell signalling is a sequence of events taking place in cells in response to changes in the extracellular environment mediated by an elaborate system of molecules present both in the extracellular milieu and various intracellular compartments.

In general, the cell signalling is initiated by a physical contact of a receptor with a stimulus followed by transduction of the signal and its intracellular propagation mediated by a chain of

intracellular molecules acting upon an effector which represents an ultimate actor modifying behaviour of the cell. During the initial step the stimulus is converted into a chemical signal represented by a change in the enzymatic activity, conformation status or concentration of certain molecules leading to propagation and distribution of the signal in the cell. The process is usually accompanied by signal amplification. The final step in the signalling cascade is modification of gene expression, releasing of metabolites, ion channel switching or a cytoskeleton change resulting in variety of cell activities such as proliferation, differentiation, secretion, a change in membrane electrochemical potential or movement (10,11).

A particular type of a cell or a particular set of cells express cell specific receptors connected to particular intracellular signalling pathways. This is a fundamental arrangement which makes it possible for a specific stimulus or combination of stimuli to provoke a specific response even though there are thousands of extracellular stimuli and thousands of cellular receptors.

Chemical stimuli sufficiently small to cross biological membranes and thus able to bind their receptors inside the cytoplasm or nucleus (NO, steroid hormones, thyroxin) or stimuli of a physical nature(light) or hydrophobic molecules do not need specific receptors on the cytoplasmic membrane. However, most of the stimuli impose their effect via binding a specific cell membrane receptor generating a chemical signal further relayed by a chain of signalling molecules until the final effector is reached. Immune signalling is triggered by interaction between the membrane-bound immunoreceptor and an antigen along with a set of co-signalling molecules (10,11).

1.2.2 Cytoplasmic membrane receptors

Cell membrane receptors are chemically proteins. Most of them can be categorised as:

1. Ion-channel-linked receptors regulating rapid signalling among excitable cells. 2. G-protein-linked receptors connected to a guanosin triphosphate-binding protein mediating the signal between the receptor and intracellular molecules by regulating enzymatic activity of other membrane-bound signalling proteins or status of ion channels. 3. Enzyme-linked receptors which either possess an intracytoplasmic component with proteinkinase enzymatic activity – receptor protein-kinases or which are connected to a proteinkinase – non-receptor proteinkinases. Receptor proteinkinases become activated by the process known as transphosphorylation

consisting in mutual phosphorylation of two peptide chains which are brought to close proximity to each other as a result of receptor cross-linking (12,13).

1.2.3 Intracellular signalling molecules

The intracellular signalling molecules chained in signalling cascades are chemically mostly proteins, but also ions, nucleotides, or lipids. It is possible to categorise signalling molecules into several functional groups. Briefly, these are relaying molecules passing a signal on the next signalling molecule in the chain, messenger molecules mediating a signal between distant proteins, adaptor proteins connecting one signalling molecule to another without conveying a signal, and transducer proteins converting a signal from one form to another. The first molecule engaged in the immune signalling cascade is a receptor anchored in the cytoplasmic membrane (12,13).

1.3. Cytoplasmic membrane

1.3.1 General structure

According to the current knowledge, the cytoplasmic membrane is a dynamic fluid structure organised in a phospholipid bilayer showing a heterogeneous chemical composition with some chemical constituents arranged in specific microdomains (14).

The cytoplasmic membrane shares the basic physical and chemical properties with other biological membranes. The main chemical constituents are phosphoglycerides (phospholipids). Phosphoglycerides are organic molecules consisting of three-carbon glycerol back-bone , two long-chain fatty acids esterified to carbons C1 and C2 representing a non-polar tail and a phosphoric acid esterified to C3 representing a polar head. Further components with similar physical properties are sphingolipids containing sphingosine as a substituent of glycerol and one fatty acid of phosphoglycerides. A subgroup of sphingolipids called glycosphingolipids is characterised by the presence of one or more sugars in their head and absence of the phopshoric acid. Further important lipid components of the cytoplasmic membrane are cholesterol, glycolipids, and triglycerides with the cholesterol being the third most common constituent. The lipids are arranged into a bilayer with the polar heads oriented outside and non-polar hydrophobic tails inside the bilayer (14).

Except for lipids, biologic membranes contain a number of proteins classified as integral membrane proteins which cross the bilayer and peripheral membrane proteins associated with the inside or the outside leaflet of the bilayer.

The peripheral proteins are bound to the lipid bilayer by several different mechanisms such as acyl chain anchoring, electrostatic interactions to membrane lipids, partial insertion into the lipid bilayer, and binding to integral proteins.

The acyl chains anchored to the bilayer can be isoprenoid, palmitoyl, myristoyl, or glycosylphosphatidylinositol tails. The myristoyl-mediated bound is weak and enforced by electrostatic interactions between polar heads of phosphoglycerides and polar side groups of the protein. Phopshorylation of the proteins can result in dissociation of the bound due to competition for the electrostatic interactions (14).

Physically, the biological membrane represents a dynamic fluid compartment with its lipid and protein components in constant movement. Only some of the constituents are fixed in their positions by interactions with cytoskeleton or extracellular domains (14).

The lipid composition varies between different biologic membranes. Within one biologic membrane, the lipids are asymmetrically distributed between the inner and the outer leaflet of the membrane. Moreover, the cytoplasmic membrane contains functionally and morphologically distinct domains specialised in nutrient absorption, cell-cell communication, and endocytosis called glycosfingolipid-enriched microdomains (GEM) or lipid rafts (14,15,16,17)

1.3.2 Lipid rafts

Electronmicroscopic studies of plasma cell membrane in 1950s revealed flask-shaped invaginations in epithelial and endothelial cells which were called caveolae. Further investigations showed their association with a family of 21-25kDa integral proteins called caveolins which represent a typical protein component creating a membrane coat overlying the caveolae. Later biochemical studies resulted in discovery of particular chemical composition and physical- chemical properties as a low buoyant density in sucrose gradient, insolubility in Triton X and a high lipid contents. Saturated fatty acids held together by hydrophobic interactions and intercalated cholesterol are responsible for insolubility and the tight arrangement into so-called liquid ordered phase. The particular physical properties of GEMs explain the ability of lateral movement in the non-raft membrane compartment arranged in disordered liquid phase (18).

Initially, there were some doubts about existence of lipid rafts in vivo as they were demonstrated only by indirect biochemical methods after membrane destruction. However, newly developed highly sophisticated techniques have made it possible to visualise them. The membrane rafts were demonstrated also outside coveolae and in lymphocytes which normally lack caveolae and caveolins (19,20).

The current view is that lipid rafts are elementary membrane microdomains freely moving laterally and clustering together in order to execute their function in endocytosis or signal transduction (21).

The lipid rafts in lymphocytes contain a number of molecules involved in immune signalling, namely co receptors CD4,CD8, adhesion receptor CD44, members of tumour necrosis factor receptor family, Src-family kinases, G-proteins, phosphatidylinsitol-4,5-biphosphate and transmembrane adaptor proteins LAT, NTAL and phosphoprotein associated with glycosphingolipid enriched microdomains (PAG) alias Csk-binding protein(Cbp) further referred to as PAG (22). Chemical composition of the membrane rafts on the one hand and location of signalling molecules in the rafts on the other hand are both essential for appropriate initiation of signal transduction. Changes in the chemical composition, e.g. depletion of cholesterol, or blocking of palmytoylation responsible for anchoring Src-family kinases(SFK) into the rafts lead to alteration of initial signalling steps resulting in either inappropriate activation of the signalling cascade or its cessation (23,24).

1.4 Src family protein kinases

1.4.1 Src family protein kinases – structure and function

An important component of the membrane rafts is a family of non-receptor tyrosine kinases – Src family-kinases (SFK).

A family of SFK proteins, further categorised into eight subfamilies, play a fundamental role in divergent cell processes as proliferation, cell-stroma adhesion and cell-cell communication.

The SFK molecule is in principle organized into six functional and structural domains (SH). A short N-terminal domain called SH4 domain is responsible for anchoring the protein in the membrane via its myristoyl and palmitoyl acid tails. Palmitoylation is a posttranslational process taking place in a membrane and it is reversible. Regulated palmitoylation and depalmitoylation can be responsible for SFK mobility in response to different stimuli. The other domains are represented by a unique region, modulating SH3 and SH2 domains, a catalytic tyrosine kinase domain, and a short C- terminal region playing a significant role in controlling SFK activity (25, 26,27).

The widespread effect of SFK is executed by phosphorylation of a number of membrane-bound or cytoplasmic molecules. Among the most important substrates of SFK are signalling molecules phospholipase C γ, RasGAP, a GTPase activator for Ras, adaptor protein Shc, and p85, a subunit of phosphatidylinositol-3-kinase, which are proteins participating in the initial signalling events followed by signal diversification and amplification. Importantly, SFK are engaged in

phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAM) of signalling subunits of multichain immunoreceptors discussed later. In turn, these provide binding sites for other SH domain-containing molecules such as SYK/ZAP 70, a subfamily of tyrosine protein kinases occupying a central position in proximal immune signalling where diverse signalling pathways start from as shown further in the text (26,27,28).

The central role of SFK in processes such as proliferation and differentiation requires tight regulation. The negative regulation of SFK activity is achieved by phosphorylation of the regulatory tyrosine residue near the C-terminus. Its phosphohorylation initiates intramolecular binding to the SH2 domain and as a result it locks the kinase in its inactive 'close'conformation which is further stabilised by intramolecular bounds between SH2 and SH3 domains. During activation the C-terminal tyrosine is dephosphorylated which results in transition into the 'open' confirmation allowing phosphorylation of the catalytic domain tyrosine (26,27).

1.4.2. C-terminal Src-kinase

The protein of utmost significance involved in negative regulation of SFK activity is C-terminal Src kinase(Csk) first described as early as 1988. Thereafter its essential role in SFK regulation has been documented in a number of studies (29,30,31,32).

According to the current knowledge, Csk is a ubiquitously expressed 50kD cytoplasmic protein sharing structural homology with the Src-kinase family possessing SH3, SH2 and C-terminal catalytic domain but not an autophosphorylation site or a C-terminal regulatory tyrosine normally present in SFK. It is presumed that active and inactive forms of Csk are in a dynamic equilibrium which is constitutively shifted towards the active state blocking activity of SFK and keeping the cell in the resting state. Mechanisms by which Csk is activated are not fully elucidated. However, recent data indicate that Csk activation is mediated by enzymatic and conformational interactions with phosphokinase A and PAG discussed later (33,34,35,36). Experimental data suggest that in its inactive state, Csk is segregated from membrane-bound SFK by binding to a cytoplasmic protein G3BP, a phosphoprotein reported to bind the SH3 domain of Ras GTPase-activating protein, located in the cytoplasm close to the immune synapse (37).

1.5 Immune signalling

1.5.1 Multichain immunoreceptors

In principle, immune signalling starts as a sequence of events initiated by immunoreceptor cross-linking by a ligand followed by phosphorylation of its signalling subunits which provide docking sites for SH2 domains of other signalling molecules transmitting the signal downstream along various pathogenetic pathways (38).

The multichain immunoreceptors are a group of multimolecular complexes comprising T- and B-cell receptors, most Fc receptors and collagen receptors expressed by platelets. They share a common structure characterised by an extracellular ligand-binding component and an intracellular non-covalently associated signalling subunit (38).

ITAMs of signalling subunits are prerequisite for initiation of the signalling cascade. The signalling subunits of a T-cell receptor complex are CD3–ζ-chains. Signalling subunits of the B-cell receptor are subunits Igα and Igβ (also known as CD79a and CD79b) (39).

It was suggested that phosphorylation of ITAMs carried out by SFK LCK and FYN in T cells and LYN or FYN in B cells mediates the connection between receptor cross-linking and subsequent initiation of intracellular signalling. The assumption was originally based on the idea that immunoreceptor complexes are associated with SFK and that upon ligation the SFK are phosphorylated via the process of transphosphorylation. According to this view the immunoreceptors should be classified as a receptor PTKs with intrinsic tyrosinkinase activity as explained above. However, it was difficult to demonstrate the association of non-raft localised T-cell receptors in unstimulated T-cells with raft localised SFK. Therefore a new concept of immunoreceptor-SFK interaction was proposed. This was based on the known membrane compartmentalisation into the raft and non-raft fractions discussed previously and it is assumed that upon receptor ligation the immunoreceptor complex diffuses laterally and merges with membrane rafts (38). However, according to some recent observations it is also possible that TCR is pre-associated with lipid rafts and the effective cross-linking leads to reorganization of the TCR complex securing ideal exposition of ITAMS to SFKs. Besides, it cannot be excluded that different T-cell sub-populations operate different TCR-raft relationships (6,40).

Despite all the current gaps in understanding the process of proximal immune signalling, it appears indubitable that membrane rafts integrity is of utmost importance as a number of signalling molecules is apparently anchored in this structure.

As discussed in the previous text, SFK are associated with the raft compartment of the cytoplasmic membrane while their major negative regulator Csk is a cytoplasmic protein. To execute its effect, Csk must be recruited from the cytoplasm to the membrane. This process is mediated by an adaptor PAG (41,42).

1.5.2 Adaptor proteins

Adaptor proteins represent a group of molecules involved in signalling but lacking any enzymatic or transcriptional activity. They role consists in organizing a molecular scaffolding for other transducers and effectors to ensure that these molecules are available for their respected tasks at an appropriate site and at appropriate time. Adaptor proteins are either cytosolic or transmembrane. These two groups differ not only in their location in the cell but also in their molecular composition (38).

1.5.2.0 Transmembrane adaptor proteins

Transmembrane adaptor proteins (TRAP) represent a recently identified group of eight molecules, with the first one (LAT) being fully characterised only in 1998 in T-cells (43) and the last one (LIME) in 2002 (44). TRAP share some similarities with signalling subunits of multichain receptor complexes, particularly with zeta and γ chains. All TRAP molecules contain a short extracellular domain and a long intracellular chain with a number of tyrosine residues called tyrosine based signalling motifs (TBSM) and serine or threonin residues which can be also phosphorylated. The phosphorylated TBSM serve as docking sites for SH2 domains of cytosolic signalling or effector molecules.

With respect to localisation in the membrane TRAP can be further subdivided into raft and non-raft-associated proteins. The raft associated adapters are PAG, LAT, NTAL, LIME while SIT, TRIM, LAX(X denotes so far unidentified cell) are representatives of non-raft TRAP. Except for PAG, the above mentioned TRAPs are expressed only in haematopoietic cells.

Despite the fact that TRAPS are not directly associated with immune receptors, with possible exception of TRIM, they become phosphorylated upon ligand-binding and their participation in recruitment of signalling molecules is necessary for cell activation (38,45,46).

1.5.2.1 LAT (Linker of activated T-cells).

The most important adaptor protein appears to be the first one identified called LAT (47). LAT is expressed not only in T-cells but in mast cells, NK cells, megakaryocytes and platelets as well. Upon ligation of the TCR, phosphorylated ITAM provide docking sites for SH2 domains of a

member of spleen tyrosine kinase family proteins (Syk) ZAP 70 (ζ-chain-associated protein kinase of 70 kDa) further phosphorylating LAT (38).

As a result of LAT phosphorylation, a multimolecular signalling complex is assembled coupling the transduction of the signal to two major signalling pathways. One is a RAS-ERK (extracellular-signal-regulated-kinase) pathway culminating in initiation of transcriptional activity of transcription factor activator protein 1(AP-1). The other pathway involves activation of phospholipase C γ generating inositol-3-phosphate (InsP3) and diacylglycerol (DAG). InsP3 induces an increase in cytoplasmic calcium concentration which activates phosphatase calcineurin finally activating transcription factor nuclear factor of activated T-cells (NF AT) whereas DAG activates phosphokinase C (PKC) which activates transcription factor nuclear factor kappaB (NFκB. Besides, DAG also induces activation of Ras-Erk kinase pathway mentioned above (48,49).

An indispensable for function of LAT is adaptor protein SLP 76. This is not only necessary for appropriate activation of PLC γ but it is also involved in activation of other molecules (38).

Due to the above mentioned protein interactions LAT was considered a positive T-cell regulator. However, recent observations that certain mutations can lead to development of lymphoproliferative disorders suggested that LAT may play a role also in negative regulation of T-cells (51).

1.5.2.2 Non–T Cell Activation Linker

Non–T Cell Activation Linker (NTAL) also known as linker of activated B-cells (LAB) was recently identified as an analogue of LAT in B-cells. It is structurally and evolutionarily related to LAT but it differs in its expression. It was identified B lymphocytes, natural killer (NK) cells, monocytes, and mast cells but not in resting T lymphocytes (51). Even though there is evidence of some functional overlapping, namely recruitment of Grb2, recent studies of LAT deficient and NTAL deficient mice showed that role of these proteins in B-cell development is asymmetrical (52). Moreover, comparisons of mast cell degranulation in wild-type and NTAL-deficient mastocytes confirmed that NTAL has different functions in initiation of mast cell degranulation and also that these two adapters differ in their topographical distribution in membrane rafts (53). Except for involvement in downstream signalling, NTAL appears to be important for internalisation of BCR after its engagement (54).

1.5.2.3 Lck-interacting molecule

Although known since 1998, Lck-interacting molecule (LIME) was first reported by Brdickova et al as a new member of the raft-associated TRAP family involved in regulation of Csk activity only in 2003 (55). LIME is expressed by T-cells. It recruits Csk to the raft region upon stimulation of co receptors CD4 and CD 8 either by antibody cross-linking or HIV gp120 binding. It was shown that LIME interacts both with Csk and SFK Lck and Fyn providing docking sites for their SH2 domains. Despite phosphorylation of the negative regulatory Tyr of LIME-associated Lck by Csk, this event does not lead to inhibition of the Lck enzymatic activity. On the contrary, the Lck becomes more active. According to the proposed explanation, the reason lies in the fact that the SH2 domain necessary for the ‚close' inactive confirmation is engaged in binding to LIME (55).

1.5.2.4 Phosphosphoprotein associated with glycosphigolipid microdomains

The other molecule involved in Csk regulation is phosphosphoprotein associated with glycosphigolipid microdomains (PAG) also known as Csk-binding protein (Cbp). The molecule was observed as an 80-kD phosphoprotein as early as 1992 but it was fully characterised and its functional role was elucidated only in 2000 by a team of investigators lead by Prof. V.Horejsi (41). The protein was called PAG. At the same time another group of investigators published similar results and called the protein Csk-binding protein (56).

Brdicka et al managed to characterise the protein on genetic and molecular level using RAJI and Jurkat T-cells showing that it is coded by a 1296 nucleotide reading frame and that is constituted by 432 amino acids. As the protein was purified from the GEM fraction it was called PAG. The protein is composed of a short, 16 amino acid extracellular domain without evidence of ligand binding properties, an α-helical transmembrane component with a palmitoylation site targeting the protein to GEM and a 397 intracellular amino acid chain. The overall structure of the protein was recognized as compatible with TRAP. It was shown that the intracellular chains contain ITAM-like sequences with 9 Tyr residues representing potential sites for phosphorylation by SFK. Moreover, the intracellular chains contain 12 Ser and 10 threonin residues that can be phosphorylated by other proteinkinases and two prolin rich sequences that may bind SH3 domains. A discrepancy was observed between molecular weight of the in-vitro phosphorylated PAG and PAG prepared from lysate of unstimulated cells which was explained by a different level of Tyr phosphorylation.

In distinction to the well known TRAP LAT, PAG was identified in a number of tissues using PAG mRNA detection by Northern blot analysis. The strongest expression was seen in immune system, heart, lungs and placenta. Localisation of PAG in cytoplasmic membrane was visualised by laser confocal microscopy using RAJI cells as a model and further confirmed by density gradient ultracentrifugation.

In vitro studies with transfection of COS cells indicated that PAG can be a substrate of Lck and Fyn but not ZAP 70 or Syk. Although in vitro studies showed association with a number of proteins involved in cell signalling, in vivo studies revealed that PAG associates only with Fyn in a way independent of Tyr phosphorylation and Csk which by contrast depends on the phosphorylation status. Experiments with mutated PAG identified Tyr 317 as a residue responsible for the interaction. Importantly, experiments showed impaired SFK activity in COS chimera cells with coexpression of PAG and Csk. Moreover, studies of human resting α/β T-cells indicated that PAG is in its phosphorylated form in resting T-cells and in its dephosphorylated form in T-cells activated by TCR ligation. It was also observed that this event is accompanied by a loss of PAG association with Csk.

It was suggested and later confirmed by other studies that in the resting T-cells PAG is present in its active phosphorylated form recruiting Csk to lipid rafts imposing its negative regulatory effect on SFKs. As PAG is supposed to be phosphorylated by SFK, the molecular system of SFK-PAG-Csk- represents a negative regulatory feedback. The phosphatase responsible for dephosphorylation of PAG is supposed to be CD45 (38, 41)

It was suggested by other authors that PAG can act as an oligomerized protein not only recruiting Csk from the cytosol to the cytoplasmic membrane but also enhancing Csk activity by modulating its conformation to increase the affinity of SFK substrates (57).

In resting T-cells, PAG is present in its phosphorylated state mediating inhibitory effect of Csk on SFK in response to ligand binding. On the other hand, studies showed that in mast cells PAG is phosphorylated only upon FcR cross-linking after its relocation to lipid rafts. Consequently, PAG recruits Csk resulting in Lyn inhibition. Presumably, in mast cells PAG acts rather as negative feedback regulator of SFK activity mitigating degranulation than as a resting state-maintenance protein (58).

Interestingly, PAG interacts with proteins other than those involved in immune signalling. It was shown that PAG albeit in low stochiometry interacts with PDZ domain of adaptor protein EBP50 further connected to actin F. It is speculated that this interaction is responsible for raft

redistribution in immune synapse formation (59). Interactions with epithelial growth factor receptor in no-haematopoietic cells were also documented (60).

The role that PAG plays in B-cells is less understood. There are only few data analysing its regulatory impact on B-cells although one of the first studies documented its possible influence on B-cell transformation. In 2003 Baumgartner et al. studied a mechanism responsible for transformation of bovine B-cells by parasite Theilleria parva which is known to induce a leukaemia-like phenotype (61). The study showed that the activation status of the cells depended on constitutive exclusion of Csk, a negative regulator of SFK, from GEMs leading to permanent activation of SFK Hck that propagates the stimulatory signals by activation of PI3K which finally leads to activation of a transcription protein AP-1. The recruitment of Csk into GEM was linked to expression of PAG whose levels were low in infected cells while in treated cells it was comparable to resting B-cells.

The regulatory role of PAG in B-cells, however, is far from clear. It seems that it may be different in B and T-cells. In B-cells SFK can induce either stimulation or inhibition. It was shown that cross-linking of B-cell receptor is associated with increased PAG phosphorylation and its Csk association which should result in decreased B-cell activity. However, Csk-PAG complex - regulated SFK Lyn, need not phosphorylate ITAMs of Ig α and Igβ chains of B-cell receptor associated signalling subunits but it can also phosphorylate ITAMs of negative regulators such as CD22 or PIRB in which case the resulting event might be positive regulation of B-cell activation by inhibition of negative regulatory pathway or negative regulation by Csk recruitment similarly to the situation in T-cells (38). This scenario would explain observations in the Theilleria parva experiment and it would be also compatible with observations of PAG expression in highly proliferating cells in germinal centres of secondary lymphatic follicles.

A large multicentre study published in 2006 showed expression of seven adaptor proteins in human non-neoplastic and neoplastic lymphoid tissue and some non-lymphoid tissue (62). PAG expression in the B-cell compartment of non-neoplastic lymphoid tissue was limited to GC of secondary follicles and marginal zones. This pattern of expression was well reflected by its expression in B-cell lymphomas. PAG was expressed in the paracortical zone and T-cell malignant lymphomas . These observations concurred with ours made in as early as 2001 (63,64). The authors suggested that adaptor proteins can potentially represent a new set of molecules suitable for routine surgical pathology practice. It will be also interesting to see whether these molecules can serve as targets for molecular therapy of ML.

Interestingly, a study conducted by Semac in 2003 (65) showed that in Burkitt's lymphoma-derived Raji cells treated by Rituximab (RTX)- anti-CD 20 antibody-based drug - CD20 redistributes into the rafts which is accompanied by lowering enzymatic activity of SFK Lyn while the composition of rafts remains unchanged and Lyn bound to its substrate is not altered. The quantity of Lyn Syk and Csk was unaffected and comparable to CD20 negative rafts. It was shown that the presence of PAG is important for Rituximab to impose its effect on SFK Lyn activity.

Two lymphoblastic cell lines known to express only a low level of PAG were treated by RTX and Lyn activity was compared to Lyn activity in RTX treated follicular lymphoma cells known to express PAG at similar level to Raji cells. Interestingly, in the lymphoblastic cell lines the Lyn activity was unaffected by RTX although RTX-dependent CD20 GEM redistribution and Lyn presence with preserved ability of autophosphorylation was detected. By contrast, in the follicular lymphoma cell line the decrease in Lyn activity was observed. The authors came to conclusion that PAG is necessary for RTX to exert its inhibitory effect. The Csk status was not studied.

Other studies using cell lines of Hodgkin lymphoma and anaplastic large cell lymphoma confirmed perturbation of lipid rafts in these neoplastic lineages and alteration of normal PAG expression (66).

The data suggest that PAG can play a part in neoplastic transformation, that variable expression of PAG in different ML entities can be used as a diagnostic marker, and that PAG may be a suitable molecule for therapeutic interventions.

1.6 WHO classification of malignant lymphomas

1.6.1 Principles

The WHO classification of haemato-lymphoid malignancies or the Pathology and genetics of tumours of haematopoietic and lymphoid tissues was published in 2001 as a long awaited guide for a unified classification of myeloid leukaemia, myelodysplastic syndrome, ML, histiocytic and dendritic cell neoplasm, and mastocytosis (67).

Classification of ML is based their histology, phenotype, genotype and clinical manifestation. The major groups are precursor and mature ML the latter comprising categories of B-cell ML, T-cell/NK-cell ML and HL.

It was possible to identify mutual counterparts of the neoplastic and physiologic B-cells which became a basis for the histogenetic approach to classification of B-cell ML as discussed later in

the text. By contrast, T-cell ML represent a less understood group of neoplasms where the histogenetic criteria were more difficult to apply.

HL represents two major types nodular lymphocyte predominant HL (NLPHL) and classical HL (cHL) traditionally divided into four subtypes – lymphocyte rich, nodular sclerosis, mixed cellularity, and lymphocyte depletion. Recent advances in molecular biologic methods particularly laser capture microdissection and PCR shed new light on the origin of HL neoplastic cells. These are now known to arise from GC B-cells with rare exceptions originating from T-cells (68-70).

ML related to the presented study are discussed in more detail.

1.6.2 Lymphoid cells and organs

Briefly, lymphoid organs are divided into primary lymphatic organs and secondary lymphatic organs. The primary lymphatic organs in mammals are the bone marrow and the thymus. The secondary lymphoid organs are the lymph nodes, the spleen, and the mucosa-associated lymphoid tissue such as Payer's patches or the palatine tonsils (71).

All lymphoid cells in adults are generated in the primary lymphoid organs – the bone marrow and the thymus.

B-cells complete their maturation in the bone marrow and leave it as mature naive B cells characterised by the germ-line Ig gene re-arrangements and production of the B-cell receptor with a unique antigen-specificity. They reside in B-zones of the secondary lymphatic organs where they are destined to undergo an affinity maturation step which starts upon encountering the appropriate antigen and leads to improvement of antigen-receptor affinity in the process of somatic hypermutation and further modification of BCR in the process of class switching.

T-cells leave the bone marrow as immature T-cell precursors. Their maturation takes place in the thymus where they undergo T-cell receptor re-arrangement and so-called thymic education leading both to the unique specificity of the T-cell receptor and ability to recognize foreign antigens exposed together with self-antigens. During maturation the T-cells acquire either CD4 or CD8 co-receptors. They leave the thymus to colonise the secondary lymphoid organs as CD4+ helper T-cells or CD8+ cytotoxic T-cells. T-cells do not undergo any further somatic mutations or affinity maturation (71,72).

1.6.2.1 B-cells

Histology and functional histology of secondary lymphoid organs is now well understood particularly as a result of enormous progress in immunohistochemistry and molecular genetic techniques. As early as 1989 there was defined a concept of composite nodule as a functional and morphological unit of the secondary lymphatic organs. The composite nodule is composed of B-cell and T-cell compartments (72).

The B-cell compartment is arranged in a primary lymphatic follicle or a secondary lymphatic follicle. The former consists of small naive B-lymphocytes and accessory cells while the latter arising as a response to naive B-cell stimulation shows a more complicated structure.

It is composed of a germinal centre (GC) and a follicle corona (72).

Several cellular types are present in the GC. Lymphoid cells are represented by large and small centroblasts histologically characterised by a large vesicular nucleus, a narrow rim of cytoplasm and one to three nucleoli attached to the nuclear membrane, centrocytes characterised by a small to medium size, cleaved nuclei, a small membrane-attached nucleolus and inconspicuous cytoplasm, and a subpopulation of T-cells. Non-lymphoid cells are follicular dendritic cells, macrophages and fibroblasts. The GC shows a typical arrangement with a recognizable dark zone comprising mitotically active centroblasts and a light zone comprising centrocytes an increased number of follicular dendritic cells. The dark zone is a compartment where a process known as clonal selection takes place. It represents the second maturation step in the development of B-cells which leads to refinement of BCR and subsequently to maturation into unique populations of plasma cells producing high-affinity antibodies or memory B-cells (72).

Antibodies are multichain plypeptides consisting of heavy and light chains each of which possesses constant and variable regions. The variable regions form an antigen binding domain with highly variable amino-acid composition organised. Variable regions of both light and heavy chains are further organised into three hypervariable regions separated by four framework regions. When the antibody molecule is assembled, the hypervariable regions also called complementarity determining regions are brought together to create the antigen-binding site (73). The Ig heavy chain constant regions determine isotype of the antibody which can be IgM, IgD, IgG, IgE and IgA. The individual isotypes differ in their functions and distribution (74).

The enormous variability of antibodies is a result of rearrangement of immunoglobulin (Ig) genes encoding variable regions of antibodies completed during B-cell development in the bone

marrow. During this process approx. 400 different genes give rise to one unique sequence of Ig genes serving as a matrix for antigen-specific antibody mRNA. Only B-cells producing functional and non-self reactive antibodies receive further survival signals and mature into naive B-lymphocytes. A corollary to the germ-line Ig gene rearrangement is that naive B-lymphocytes leaving the bone marrow bear a cell-unique BCR of IgM or IgD isotype designed to recognize one specific antigen (74).

The antigen-affinity is further refined in the process of somatic hypermutation representing the second maturation step which takes place in the germinal centres. Only cell producing high affinity BCR are positively selected and mature into effector cells – plasma cell and memmory cells. The key events of somatic hypermutation are point mutations, usually single-nucleotide replacements within the Ig variable region genes occurring in a very high rate. As a result, some cells produce BCR with affinity to the immunising antigen superior to BCR produced by other cells. The former receive survival signals while the latter die by apoptosis (74). Therefore preserved BCR signalling machinery is a prerequisite for the fate of B-cells during immune response which is a feature preserved to a certain extent in some malignant lymphomas.

Another important step modifying the BCR during GC transition is class switching. It is a process affecting the Ig heavy chain constant region genes during which IgD and IgM are looped out and Ig variable joining sequences become associated with the downstream genes encoding γ, ε and α chains. The result is production of IgG, IgE or IgA antibodies. The class switching leads to changes in function of the secreted antibody but not to changes in antigen-binding specificity (74).

The above described processes taking place in the bone marrow and germinal centres are important for oncogenesis as they are inherently associated with DNA breaks whose resolution can lead to errors responsible for generating malignant phenotype (75,76).

The GC is surrounded by the follicle corona consisting of two zones.

The inner zone is the follicle mantle composed of small naive lymphocytes characterised by the germ-line Ig gene re-arrangement and no somatic variable Ig genes mutations. These are resting B-cells waiting for stimulatory signals after which they enter GC. In many organs particularly the spleen, the intra-abdominal lymph nodes, Payer's patches and the palatine tonsils there is a second zone, the marginal zone lying outside the mantle zone. It is marked by the presence of so called monocytoid cells or marginal zone cells comprising a pool of memory B-cells (72). Some

memory cells experienced transition through GC and these are characterised by somatically mutated Ig variable region genes and CD27+ expression. However, studies of human spleens suggested that memory B-cells with mutated Ig genes can be generated outside GC possibly as a maturation program of B-cells prior to encountering an antigen (77).

Immunohistochemical studies identified molecules associated with the B-cell origin. These are CD 19, CD22, CD20, CD79a, the latter two are easily detected in paraffin sections while the former are used as B-cell markers in flow cytometry studies (72).

The T-zones of the composite nodule correspond to paracortical zones of the lymph node. They are intimately associated with lymphatic follicles. Their major components are small T-cells with prevailing CD4 positive helper T-cells over CD8 positive cytotoxic T-cells. Lymphocytes enter the paracortical zone through specialised small vessels called high-endothelial venules. Without stimulation T-cell leave the compartment via efferent lymphatics. If they encounter receptor-specific antigen presented by the interdigitating dendritic cells, they become activated and activated T-cell initiate activation of B-cells and macrophages. The T-cells are characterised immunohistochemically by expression of T-cell-associated markers- CD2 and CD3 (72).

1.6.2.2 B-cell malignant lymphomas

Numerous immunohistochemical and molecular biologic studies of normal lymphoid tissue and malignant lymphomas revealed that the malignant lymphomas share not only morphologic but also immunophenotypic and genetic similarities to cells occupying the well-defined compartments of lymphoid tissue (67).

B-cells leaving the bone marrow are naive CD5+ B-cells expressing surface IgD and IgM and lacking CD27. They are believed to give rise to mantle cell lymphomas (MCL) and a proportion of chronic lymphocytic leukaemias (CLL/SLL). The morphologic, immunophenotypic and functional properties are marked by the small cell morphology, CD 5 expression, germ-line Ig genes rearrangement and tendency to develop a disseminated disease (67,75,76).

There is mounting evidence that GC is a source of several types of malignant lymphomas both of a small cell type, indolent, and a large cell type, aggressive.

The former group is represented by the entity of follicular lymphoma (FL). This is composed of normal B-cell lymphoid constituents of the germinal centre - centroblasts and centrocytes. The shared immunophenotype with the reactive GC cells is characterised by CD 10 and bcl-6

expression and the pathogenetic pathway by aberrant bcl-2 expression, a result of t(14,18) translocation. The latter group is represented by a diffuse large B-cell lymphoma and Burkitt's lymphoma (75,76,78).

As my study is focused on PAG expression in DLBCL, more detailed discussion follows.

Diffuse large B-cell lymphoma is the most common lymphoma constituting 30 to 40% of all non Hodgkin lymphomas in Western countries (67). Morphologically it is subdivided into four types which are centroblastic, immunoblastic, T-cell/histiocyte rich and anaplastic ML with two rare variants – plasmablastic DLBCL and DLBCL with full-length ALK. Mediastinal large B-cell lymphoma, intravascular large B-cell lymphoma and primary effusion lymphoma are classified as separate entities (67).

Morphologic heterogeneity of DLBCL subtypes does not reflect the variable course of the disease and responsiveness to therapy. Instead, it was shown that there are other distinguishing markers more successfully subclassifying DLBCL into clinically relevant subtypes (79).

The major breakthrough followed introduction of gene expression profiling into investigatory armamentarium. Using this method it was possible to identify three different types of DLBCL – GC type, activated B-cell type and unclassified type 3, now grouped into two DLBCL subsets – GC-like and non-GC-like or activated DLBCL differing in their gene expression profile and most importantly in clinical outcome (80).

The differences found at the gene expression level or mRNA level were later confirmed by correlation with chromosomal aberrations detected by comparative genomic hybridisation (81). The different gene expression profiles and chromosomal aberrations in these two subsets are reflected by differences in oncogenic pathways as documented in a number of studies. With regard to the biologic and clinical differences it was suggested that these two subtypes represent two separate diseases (79-84).

Alternatively, a classification of DLBCL based on the immune host response- and micro-environment-induced gene expression profiles also identified clinically relevant DLBCL subtypes which are likely to be responsive to different therapeutic interventions (85).

The GC-like DLBCL subtype is presumed to arise from GC B-cells. It is characterised by expression of genes transcribed in normal GC cells, particularly bcl 6. Bcl 6 is a master repressor gene blocking plasmacytic differentiation mainly by downregulation of another master gene BLIMP 1, normally orchestrating plasma cell differentiation, and upregulation of p27, a negative

regulator of cell cycle progression. Consequently, the plasma cell differentiation program is switched of and the transformed germinal centre cells remain in cell cycle (86-7).

The pathogenic event detected in 45% of GC-like DLBCL but not in non GC-like DLBCL is BCL-2 dysregulation as a result of t(14;18) translocation which brings BCL-2 gene on the cromosome 18 under regulatory influence of the Ig heavy chain gene enhancer on the chromosome 14. The translocation results in bcl 2 protein overexpression imposing its anti-apoptotic effect and favouring survival. As BCL-2 gene is not transcribed in normal GC cells, this translocation provides a selective advantage in the process of neoplastic transformation of GC cells which can result in GC-like DLBCL (84).

Some studies suggested that amplification of c-rel locus is another typical feature of GC-like DLBCL. This is, however, not followed by up-regulation of NFκB target genes in this lymphoma type. Significance of this finding for tumour progression is still uncertain (80,88).

Finally, there were demonstrated on-going IgH mutations consistent with GC cell derivation in this DLBCL subtype (89).

Although the origin of non-GC-like DLBCL is less clear, this lymphoma is supposed to arise from B cells poised to exit the GC and differentiate into plasma cells. This concurs with its gene expression profile which is similar to mitogen-activated peripheral B cells and the cells undergoing plasmacytic differentiation (79).

The principal pathogenic event in non-GC like DLBCL is activation of NFκB B pathway due to constitutive activity of IκB kinase resulting in expression of NFκB target genes. This sequence of events is not observed in GC-like DLBCL (82). In contrast to GC-like DLBCL, non-GC-like DLBCL has a fixed range of immunoglobulin gene mutations, suggesting that the somatic hypermutation machinery has been inactivated (88).

Bcl 2 is expressed in a proportion of non-GC-like DLBCL but due to different alterations than t(14,18) translocation, possibly due to transcriptional deregulation of bcl-2 in part as a consequence of NFκB activation or BCL 2 gene amplification (88).

A recurrent structural abnormalities- mutations and deletions and epigenetic silencing observed in a proportion of non-GC like DLBCL cases but not in GC-like cases involves Blimp1 gene, a tumour suppressor gene normally orchestrating plasma cell differentiation. However, BLIMP 1 gene is not expressed in GC-like DLBCL, which concurs with GC cell derivation. Its inactivation

in GC is explained by activity of bcl 6 and not by chromosomal aberrations or epigenetic silencing (83).

The substantial differences in oncogenic mechanisms can have a practical impact on therapeutic strategies in foreseeable future. NFκB blockers are promising targets for non GC-like DLBCL but different drugs may be required for treatment of GC-like DLBCL (90).

These prospects drive research both into tumour-specific drugs and diagnostic procedures. It is very likely that shortly It will be a routine task for practicing pathologists to subclassify DLBCL.

Gene expression profiling, though a powerful method for DLBCL subclassification, is not still available as a routine method in most of clinical laboratories providing haematopathological services. Therefore, there were conducted studies in order to find surrogate markers easily detectable by use of routine immunohistochemistry.

As the gene expression profiling implicated that the GC-like subtype of DLBCL is derived from GC cells while the non-GC-like subtype from post-GC B cells undergoing plasma cell differentiation , expression of reliably and reproducibly detectable GC and plasma cell-associated antigens CD 10, bcl 6, MUM1 and CD138 were tested as possible surrogate markers of DLBCL subtypes (91,92).

CD10 (CALLA, common acute lymphoblastic leukaemia antigen, neutral endopeptidase-24.11, EC 3.4.24.11, NEP, encephalokinase, neprilysin) is a 94 kDa zinc-dependent cell membrane metalloprotein that was proved to be a reliable marker of GC cell differentiation in a number of studies. CD 10 is expressed in early lymphocyte development, down-regulated later and re-expressed again upon entry in the pool of GC B-cells. This expression pattern corresponds to expression in malignant lymphomas. It is expressed in precursor B-lymphoblastic leukaemia/lymphoma and in GC cell derived malignant lymphomas such as Burkitt's lymphoma, FL, and in a proportion of DLBCLs, usually together with bcl-6 protein. Moreover, CD 10 was found to be a prognostic marker in DLBCL in some studies (67,78,93).

Bcl-6 protein is a POZ/zinc finger transcription factor acting as a transcriptional repressor. It is indispensable for GC development where it blocks plasma cell differentiation.. Bcl 6 expression in malignant lymphomas reflects its physiological expression as it is regularly detected in GC cell derived malignant lymphomas similarly to CD10. It is supposed that bcl 6 contributes to lymphomagenesis by blocking BLIMP 1 expression discussed above. Except for B-cell

lymphomas and GC cells, bcl 6 expression is documented in CD30 positive T-cell lymphomas and CD30 positive non-neoplastic cells in the interfollicular region (94-96).

MUM1 is an antibody developed against a product of IRF4 gene. In normal cells IRF4 is expressed in late developmental stages of GC cells and plasma cells. Its expression is mutually exclusive with bcl-6 expression. Their co-expression is, however, well known in a proportion of diffuse large B-cell lymphoma cases where this is interpreted as evidence of post-GC cell differentiation which is why this is used as a marker of non-GC DLBCL subtype. IRF4 plays an oncogenic role in myeloma oncogenesis where its overexpression is a result of t(6;14) translocation juxtaposing MUM1/IRF4 gene to heavy IgH gene (97).

CD138 is a transmembrane heparan sulphate proteoglycan functioning as an adhesion molecule expressed in epithelial cells and in terminal stages of B-cell differentiation. It is acquired as the GC cells differentiate towards plasma cells. This makes it possible to use CD 138 as a post-GC immunohistochemical marker expressed in non-GC DLBCL subtype (98).

Gene expression profiling indicates that the sub classification of DLBCL into GC and non-GC-like subtypes bears prognostic information independent of other prognostic variables (79). The findings were supported by quantitative real-time PCR analysis of a set of six genes where those belonging to the GC B-cell signature correlated with longer survival while the genes belonging to activated-B cell signature in microarray analysis correlated with shorter survival (99).

A number of molecules other than those associated with GC and plasma cell differentiation were tested as DLBCL prognostic markers. These included cell cycle regulators as cyclins , anti-apoptotic proteins as bcl 2 or other B-cell differentiation markers as FOXP1 (100).

The most robust prognostic variable - the International Prognostic Index (IPI) is based on clinical and biochemical data - extent of the disease, lactate dehydrogenase serum levels, patient's age and performance status. Using IPI, the lymphoma patients can be stratified into four subgroups – low risk, low-intermediate risk, high-intermediate risk and high-risk according to their outcome - with a very poor (26%) or a good (73%) 5-year overall survival (101). Its predictive value can be improved if it is combined with some immunophenotypic characteristics associated with either GC-like or non-GC-like DLBCL subtypes, as bcl 2, CD10 or FOXP1.

Combination of IPI and bcl-2, known to be associated with unfavourable outcome in DLBCL patients, allowed investigators to classify a large proportion of intermediate prognostic group as high-risk patients (28%). According to the authors, these patients might benefit from a different

therapeutic regime than patients in the other groups (102). By contrast, combination of IPI with CD10 in another study identified patients with extraordinary good survival (92% in 8 years) while bcl-2 in the high-risk group identified patients with a very dismal prognosis and overall survival less than 14 months (103). Although there are conflicting reports, two most recent studies showed that FOXP1, a transcription factor broadly but variably expressed in variety of tissues including solid tumours (104), is a robust differentiation and prognostic marker. It is known to be expressed in activated B-lymphocytes and its strong and diffuse reaction pattern showed high concurrence with non-GC-like phenotype according to a study demonstrating that 91% of 23 FOX P1 positive cases displayed non-GC-like phenotype (105). Moreover, a recent report analysing data gathered from 101 DLBCL patients confirmed previous observations that FOX P1 is a prognostic factor independent of IPI index (106).

Another GC cell derived malignant lymphoma is Burkitt's lymphoma. This is characterised by t(8,14) translocation in most of the cases resulting in MYC translocation from chromosome 8 to IgH gene region on chromosome 14 (67, 76).

As recent studies have shown, Hodgkin lymphoma is also a GC derived neoplasm. This fact concurs with known CD20 and CD79a expression in a significant proportion of cases (67,70).

The mantle zone is a site of origin of mantle cell lymphoma (MCL). Most of the cases are composed of small cells but blastoid variant exists and it is believed this is a result of additional mutations to the hallmark t(11,14) translocation with cyclin D1 overexpression (67,76).

The group of marginal zone lymphoma embraces three entities – extranodal marginal zone lymphoma (MALT type), nodal marginal zone lymphoma and splenic marginal zone lymphoma sharing morphologic similarity with the monocytoid cells of the marginal zone but being a group of separate entities (67,107-8).

New light has been recently shed on chronic lymphocytic leukaemia/small lymphocytic lymphoma (CLL/SLL). Although this ML shows only minor morphologic differences among individual cases, there is marked variation in course of the disease (67). The patients can experience either a long-term survival or die few months after the diagnosis is established. Gene expression profiling showed correlation between biologic behaviour and mutation status of the variable IgH regions. The mutated genotype suggesting that the neoplastic cells are derived from a precursor that experienced passage through the GC correlates with more favourable survival than observed in patients without evidence of Ig gene mutations (109-110). A number of studies

were conducted to find a single or an acceptably limited number of markers easy to demonstrate by routine immunohistochemistry in order to differentiate between these types of CLL/SLL.

The most informative molecule so far turned out to be ζ-chain-associated protein 70 known as ZAP 70. Although ZAP 70 is not detectable by immunohistochemistry in mature non-neoplastic B-lymphocytes, it is re-expressed in CLL/SLL where its function is unknown but presumably it participates in immune signalling mediated by BCR in the unmutated CLL/SLL type (111).

ZAP 70 is a signalling molecule playing a central role in proximal T-cell signalling (112). Its role is well documented in T-cells but much less is known about its function in B-cells where it is supposed to be involved in early stages of development but later it is apparently lost from the proteome as it is not expressed by mature B-cells leaving the bone marrow. The suggested mechanism of T-cell activation presume that upon ligation of T-cell receptor, ITAMs of T-cell receptor signalling subunits are phosphorylated subsequently providing docking sites for ZAP 70. Upon being recruited to close proximity of SFK, ZAP 70 is phoshorylated and together with SFK it participates in phosphorylation of various substrates, e.g. adaptor proteins LAT and SLP76 discussed above (38).

1.6.2.3 T-cells

T-cells that underwent maturation and education in the thymus are released to circulation and reside in T compartments of the composite nodule in lymph nodes represented by the paracortex. The T-zones shows a less defined micro-architecture than the B-cell lymphatic follicle. T-cells can form either a well defined nodule or just an ill-defined lymphocytic aggregate (72).

Effector functions of T-cells depend on interactions with foreign proteins expressed on the surface of cells. T-cells do not react with soluble antigens. They either kill the target cells directly or activate other cells involved in immune response such as macrophages and B-cells. For the latter task they need collaboration with antigen presenting cells particularly dendritic cells, B-cells, and macrophages.

T-cells are specialised in recognition of antigens bound to major histocompatibility molecules which are expressed on the surface of other cells, e.g. interdigitating dendritic cells of the paracortex, and which also express other co-stimulatory molecules significantly enhancing the activation process as CD80 and CD86 - ligands of CD28 expressed by T-cells (72,113).

T-cell receptor (TCR) is similar in structure to antibody binding fragment of BCR. It is a heterodimer composed of two chains with variable- and constant-like regions. Brought together the variable parts form complementarity determining regions or antigen binding sites. TCR does not exist in a soluble or secretory form; it is anchored in the cytoplasmic membrane by a transmembrane region. The cytoplasmic tail is short and successful signal transduction mediated by TCR requires association with signaling subunits which are ζ chains of CD3 molecular complex associated with TCR (38).

The peptide chains of T-cell receptor can be either α/β or γ/δ chains. The α/β TCR-bearing T-cells predominate being responsible for most of T-cell mediated immune responses which are essentially influenced by interactions of co-receptor molecules either CD4 or CD8. The γ/δ T-cells represent a less numerous population of more primitive cells which are present particularly in the epithelium of the gastrointestinal tract and the red pulp of the spleen. They differ from α/β T-cells both immunopenotypically lacking CD4 and CD8 and functionally as they are involved mainly in mucosal defence and in the immune response to mycobacterial infection and their ability to recognize antigens is not MHC restricted. (71,113-4).

During the T-cell development in the thymus T-cells undergo immunophenotypic maturation so that immature subcapsular CD4 and CD8 positive cells express only one of these co-receptors when reaching the medulla. Consequently, T-cells can be divided into functionally different populations CD 4+ T-cells called helper cells and CD8+ T-cells called cytotoxic cells. The former can be further classified according to their functional specialisation as Th1 and Th2 cells. Th1 cells secrete IL 2 and interferon γ and not interleukin 4, 5, and 6. Their main role is to serve as ‘helpers‘ for other T-cells and macrophages in particular. By contrast, Th2 T-cells secrete interleukins 4,5, 6, and 10 and they act as ‘helpers‘ of B-cells (113-4).

The cytotoxic CD8+ cells are involved in cytotoxic reactions. They are potent secretors of perforin, granzyme B and TIA 1 all being executors of cell-killing. They share some common features with NK-cells which express T-cell markers as CD2, CD7, CD8, CD56, CD57 and cytoplasmic CD3 reflecting the presence of CD3-associated ε-chains (71, 72,113-4).

1.6.2.4 T-cell malignant lymphomas

Despite the above described conception of T-cell development and sub-specialisation represented by CD4+, CD8+ and NK populations, T-cell malignant lymphomas differ from each other rather clinically then phenotypically as morphological and immunophenotypic features are often overlapping (67).

As a result, most of the T-cell lymphomas in routine practice are classified as a peripheral T-cell lymphoma, not otherwise specified. Only a lesser number of cases represent morphologically and Immunophenotypically defined categories as angioimmunoblastic T-cell lymphoma or anaplastic large cell-lymphoma (67).

It can be a diagnostic challenge to recognize a neoplastic population in routine diagnostic practice as there is no single immunohistochemical marker analogous to cyclin D1 helpful in diagnosing mantle cell lymphoma or bcl-2 lending support to follicular lymphoma in the differential diagnosis with follicular hyperplasia. Even PCR demonstration of clonality by analysing TCR rearrangement can fail to disclose clonal population due to a large number of consensus primers needed for the reaction if β-chain is tested. Therefore new PCR techniques were recommended to overcome the problem (115-6). There are also no known typical recurrent genotypic abnormalities that can be helpful in the diagnostic process except for t(2;5) and its variants characteristic of anaplastic large cell lymphoma (67,117).

Clinically, T-cell lymphomas tend to follow more aggressive course than B-cell malignancies (118-9).

One of the reasons why T-cell lymphomas are less understood is their relative rarity particularly in Europe and North America but even in the worldwide perspective. T-cell neoplasms represent only 12% of non-Hodgkin lymphomas (67).

1.6.2.5 Hodgkin lymphoma

When described in the late ninetieth of the 19th century, Hodgkin lymphoma was considered to be an inflammatory disorder. Even after it was evident that the disease is a neoplasm rather than a reactive disorder, it was difficult to track the neoplastic cells as these are much less frequent than the non-neoplastic background. Summarising morphology, immunophenotype and clinical data it became obvious that Hodgkin lymphoma is a heterogeneous disease representing two separate entities having in common scarcity of morphologically similar but not identical neoplastic cells which are present in the inflammatory background differing in its quality. These major subtypes are nodular lymphocyte predominant Hodgkin lymphoma and classical Hodgkin lymphoma (67).

Intensive research in the last decade of the 20th century was accomplished by elucidation of an origin of the neoplastic cells which are popcorn or lymphocytic and/or histiocytic Reed-Sternberg cell variant (L&H) in NLPHL and Hodgkin and Reed-Sternberg cells (HRS) in cHL.

The ultimate breakthrough in characterisation of L&H and R-S cells was a corollary to enormous progress in development of molecular genetic techniques which made it possible to investigate genetic alterations in single cells. The cells were isolated by laser capture microdissection from

frozen sections and analysed by polymerase chain reaction (PCR) which showed clonal rearrangement and somatic mutation of Ig variable regions genes indicating their derivation from GC cells. Differences in mutational status between NLPHL and cHL were observed. Intraclonal Ig gene variability in L&H cells indicated on-going Ig gene mutations and thus derivation from proliferating centroblasts whereas crippling Ig gene mutations in R-S cells indicated the origin in pre-apoptotic B-cells which were rescued from apoptosis possibly due to a concurrent loss of B-cell lineage associated markers (120-121). Only rare cases appear to be of a T-cell origin as suggested by clonal TCR γ and β rearrangements (122-123).

In some instances, differentiation of cHL from NHL is uncertain due to overlapping morphologic and immunophenotypic features. of the neoplastic cells (124-6). However, authors of a recent study were able to differentiate between these so-called grey-zone lymphomas analyzing the whole tissue samples containing both the neoplastic cells and the reactive background. They demonstrated a unique gene expression profile of classical Hodgkin lymphoma separating this entity from non-Hodgkin lymphomas (127). The results are in agreement with the textbook paradigm that the mere presence of cells displaying cytomorphology of R-S cells is not sufficient for the diagnosis of Hodgkin lymphoma as these must be demonstrated in the typical background before the diagnosis is established.

As the principal differences in biology of L&H and R-S cells implicate differences in immune cell signalling whose integral component is PAG, more detailed discussion follows.

Nodular lymphocyte predominant Hodgkin lymphoma is defined by nodular architecture and presence L&H in a small lymphocytic background occupying a hyperplastic meshwork of follicular dendritic cells. The L&H cells possess a distinctly deformed plump pale nucleus with a less prominent nucleolus than in R-S cells. They are surrounded by a collaret of T-lymphocytes expressing CD57 normally detectable on intrafollicular T-cells. Immunophenotypically, the L&H cells express LCA (common leukocyte antigen) and B-cell markers as CD 20, CD 79a, bcl 6 and transcription factors Bob2 and Oct1, PU.1, and in approx. 50 % of cases EMA (epithelial membrane antigen). CD15 and CD 30 are not expressed and EBV is not detected (67).

The patients are middle aged males. The disease affects superficial lymph nodes and at the time of diagnosis it is usually a localised disease. The outcome is in general favourable. A specific condition called progressive transformation of GCs is supposed to be pathogenetically related to the development of the NLPHL (67).

The classical Hodgkin lymphoma differs both morphologically and clinically from NLPHL. The neoplastic cell – Reed-Sternberg (R-S) cell - is notoriously known as a large cell possessing overlapping multiple nuclei or a bilobed nucleus with prominent oxyphilic large nucleoli surrounded by a distinct halo. Except for the classical or diagnostic R-S cells, there are two other

variants. One is represented by lacunar cells displaying a remarkable artefact resulting from shrinkage of the cytoplasm and leading to peculiar lacuna-like formations around the shrunken cell. The nucleus harbours a less prominent nucleolus than the diagnostic R-S cell. The other R-S cell variant is the Hodgkin cell characterised by one large nucleus with other features typical for R-S cells (67).

Variations in morphology of the neoplastic cells and in the inflammatory background serve as a basis for division of the cHL into four categories. These differ to some extent in clinical manifestations such as a site of involvement and a course of the disease. Names of the subtypes are descriptive – lymphocyte rich, mixed cellularity, nodular sclerosis and lymphocyte depleted. The immunophenotype of R-S cells is unique. The R-S cells lack expression of B-cell receptor and usually B-cell associated markers but express typically CD 15, CD30, CD95 and PAX5 (paired box gene 5 encoding B-cell lineage specific activator -BSAP). Weak CD 20 expression can be seen in approx. 30% of the cases but LCA and CD 79a are typically negative. Negativity of EMA is an important diagnostic marker in the differential diagnosis with NLPHL (67).

The R-S cells do not synthesize immunoglobulins even at the transcriptional level (70). As explained above, B-cell receptor synthesis is a critical step in the process of positive selection or survival during clonal expansion in the GC. Non-selected cells are prone to die by FAS receptor-mediated apoptosis. Therefore the balance of pro- and anti-apoptotic mechanisms in R-S cells must be shifted towards cell survival Two major mechanisms which appear to be mutually interconnected were suggested. These are sustained activity of R-S cells and protection from apoptosis due to alterations in various signalling pathways (70).

The sustained activity can be initiated by the following events - gene amplification of c-rel, ligand-mediated and ligand-independent activation of cell-surface receptors such as CD30, CD40, receptor activator of NFκB (RANK), or NOTCH1, EBV-encoded late membrane protein 1 (LMP1) or LMP2a in EBV-positive cases and loss-of-function mutations of the tumour suppressor IκBα. Although several pathways are activated, sustained activation of NFκB appears to be the most important and consistently activated pathway (70,128).

The prevention of apoptosis involves inhibition of both extrinsic and intrinsic activation of the caspase cascade. The anti-apoptotic effect is likely mediated by the major anti-apoptotic protein FADD-like interleukin-1ß-converting enzyme-inhibitory protein known as c-FLIP which inhibits the Fas receptor-induced extrinsic pathway and by

overexpression of X-linked inhibitor of apoptosis (XIAP) which interferes with the intrinsic pathway. While it is supposed that c-FLIP overexpression is a result of NFκB activation, the XIAP effect appears to be NFκB independent (70).

A novel epigenetic mechanism affecting primary mRNA transcript has been suggested recently as a possible primary oncogenic event. It is based on the observation of post-transcriptional alterations of transcription factor blimp-1 mRNA in R-S cells by activity of small RNAs. The mRNA alteration leads to inactivation of blimp-1 protein, necessary for orchestration of plasmacytic differentiation under normal conditions. Authors suggested that the block in differentiation of GC cells into plasma cells contributes to neoplastic transformation (129).

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2.0 PAG in Haematopathology – Objectives and Theses

ML with variable clinical outcomes difficult to explain by their phenotype represent logical subjects for investigation. A neoplastic entity defined by conventional criteria can show significant variablity when analysed by molecular genetic methods. Differences at the chromosomal level or the gene expression level are reflected by an abnormal protein composition affecting basic cellular functions such as regulation of survival or secretion. Identification of such differences is important from therapeutical point of view as dysregulated molecules can be therapeutically targeted by molecule-specific drugs. Along with introduction of the molecule targeted therapy, it is necessary to formulate reliable and reproducible diagnostic criteria applicable in routine diagnostic services in order to identify tumours suitable for the therapy.

One of the outstanding achievements in the field of tumour-targeted therapy is drug Gleevec, a protein kinase inhibitor which is able to suppress activity of several protein kinases such as v-Abl, PDGF receptor, and Kit receptor by blocking their active sites. The drug is successfully used in treatment of chronic myeloid leukaemia with recurrent abnormality t(9,22) and the gastrointestinal stromal tumours (4,129). Another approach to the tumour-targeting therapy is based on the detection of cell specific antigens. An example is Rituximab, an anti CD 20 antibody used in the therapy of some NHL (130). New potential targets for therapeutic agents are being investigated. Progress in understanding of the cell signalling and cell cross-talking in oncogenic processes drew attention of invetigators to signalling molecules involved in lymphoproliferative disorders (131-2) and some of the molecules may turn out useful diagnostic markers (133-4).

PAG is one of the transmembrane adaptor proteins playing an important role in physiological regulation of lymphocyte activation as it is a significant component of the SFK regulatory loop. Despite its importance under physiological conditions, there had been very limited data available on PAG expression in human non-neoplastic and neoplastic lymphoid tissue before I commenced my study. Therefore objectives of my study were to determine expression pattern of PAG/Cbp in lymphoid tissue, to identify possible specific associations with ML entity(ies), and to find out whether PAG detection could be useful in routine diagnostic haematopathology.

First I conducted a pilot study and then formulated theses based on its results.These were as follows:

- PAG is expressed in FL but not in MCL and CLL/SLL

- PAG is expressed in GC-like DLBCL but not in non-GC-like DLBCL
- PAG is expressed in L&H cells but not in R-S cells

In case the theses were proved true, PAG could be considered an adjunct in the differential diagnosis of ‘small cell lymphomas’, Hodgkin lymphoma and sublassification of DLBCL in particular.

3.0 Results:

The most optimal antibody (MEM 255) was identified among five monoclonal antibodies raised against PAG in the laboratory of Prof. V. Horejsi,UMG, Praha, (results are not shown). Then PAG expression was studied in non-neoplastic and neoplastic lymphoid tissue in a pilot study. Its results were analysed and arising issues were eventually addressed in a subsequent study of a larger series of ML.

Manuscripts of the studies as accepted for publication are re-printed below.

Expression pattern of adaptor protein PAG: correlation between secondary lymphatic follicle and histogenetically related malignant lymphomas.

Alexandr Svec, Zuzana Velenska, and Vaclav Horejsi

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Short communication

Expression pattern of adaptor protein PAG: Correlation between secondary lymphatic follicle and histogenetically related malignant lymphomas

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Abstract

Transmembrane adaptor protein PAG, also known as Csk-binding protein (Cbp), which binds and activates the cytoplasmic tyrosine kinase Csk, the major negative regulator of Src-family kinases, was found to be expressed in germinal centers of lymphoid follicles as well as in follicular, but not mantle cell lymphomas. Expression of PAG may reflect its role in regulation of proliferation and differentiation of germinal center B-cells. From the routine histopathology point of view, PAG might be a new positive marker of follicular lymphoma and a negative marker of mantle cell lymphoma.

Keywords: Germinal center; Lymphoma; PAG; Histopathology

1. Introduction

Phosphoprotein associated with glycosphingolipid enriched microdomains (PAG), also known as Csk-binding protein (Cbp), is a broadly expressed transmembrane adaptor protein present in membrane microdomains called membrane rafts or glycosphingolipid enriched microdomains [1,2]. Tyrosine phosphorylated PAG binds a tyrosine kinase Csk, the major negative regulator of Src-family kinases [1–7]. This brings the cytoplasmic Csk to the proximity of its membrane-localized substrates; furthermore, complexing with PAG increases the intrinsic activity of Csk [6]. In resting peripheral blood T lymphocytes, relatively high level of the phospho-PAG/Csk complex appears to suppress activity of Src-kinases Lck and Fyn, and thereby helps to set an activation threshold in these cells. Following ligation of T-cell receptor (TCR), PAG becomes dephosphorylated which leads to release of Csk and activation of the Src-kinases; this may play a role in the onset of T-cell-based immune responses [1,3,7,8]. Suppression of PAG expression may participate in pathologic B-cell lymphoproliferation, as recently demonstrated for theileriosis [9]. Therefore, it may be expected that PAG expression (and phosphorylation) may participate in regulation of lymphoid cell proliferation and differentiation, and that is why we wished to determine the PAG expression in various compartments of secondary lymphatic follicles and in lymphomas derived from them. Our present data demonstrate that the protein is strongly expressed in germinal centers of lymphoid follicles and also in follicular, but not mantle cell lymphomas.

2. Materials and methods

Thirty malignant lymphomas diagnosed in the Department of Pathology, Faculty Hospital Královské Vinohrady, Prague, since May 2001 to March 2004 were tested for the

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PAG expression. The diagnoses were established according to the WHO classification [10] after consensus of at least two pathologists on the basis of histological features and immunohistochemical profiles. Diagnoses of five mantle cell lymphomas were further confirmed by PCR and/or FISH detection of *t*(11; 14) translocation and by flow-cytometric immunophenotyping in two other cases. In addition, one superficial and four deep lymph nodes displaying follicular pattern of hyperplasia and an appendix with hyperplastic lymphoid tissue were included in the study.

Tissue samples were formalin fixed and routinely processed. The immunohistochemical reaction included an antigen retrieval step heating in citrate buffer pH 6.0 for 20 min. Mouse IgG2a monoclonal antibody MEM-255 (Exbio Praha, Czech Republic) was prepared from a mouse immunized with recombinant human PAG as described elsewhere [1]; it reacts optimally with denatured PAG (under the conditions of Western blotting) whether tyrosine phosphorylated or not. MEM-255 was used here for the tissue section immunostaining as a primary antibody. The reaction was visualized by the Universal LSAB+ kit (DAKO, Carpinteria, CA) according to the manufacturer's recommendation.

The samples were considered positive if the brown reaction product clearly highlighted cytoplasmic membranes in a distinct linear pattern in more than 10% of the neoplastic cells. Each reaction was compared to the negative control treated in the same procedure, but the primary antibody was omitted.

3. Results

In hyperplastic lymphatic tissue, the germinal centres of secondary lymphatic follicles were strongly positive with the PAG-specific mAb MEM-255, clearly contrasting with essentially negative small lymphocytes in the follicle mantle that contained only scattered clearly positive cells whose distribution correlated with $CD3^+$ T-cells (Fig. 1A and B and data not shown). The paracortical T-cell area displayed a weak positive reaction. Malignant lymphomas followed the same pattern of expression: follicular lymphomas (four cases grade 1, four cases grade 2, one case grade 3) were strongly positive (9/9) highlighting neoplastic "germinal centers" (Fig. 1D), whereas mantle cell lymphomas (7/7) (Fig. 1C), small lymphocytic lymphomas (3/3), and extranodal marginal zone lymphomas (3/3), plasmocellular neoplasms—plasma cell myeloma and plasmocytoma (2/2) were completely negative (not shown). Diffuse large B-cell lymphomas (DLBCL) were heterogenous—two cases were positive and three negative. Classic Hodgkin lymphomas (7/7) were PAG-negative (not shown). The results are summarized in the Table 1.

4. Discussion

The strong expression of PAG in proliferating cells of germinal centers and in follicular lymphomas derived from these cells is in striking contrast to the expectation based on the assumption that PAG participates at negative regulation of Src-kinases (thus, proliferating cells should express little PAG in order to keep the Src-kinases active). Similarly, surprising is the observed lack of PAG expression (or at least substantially lower expression not detectable under the conditions used) by mantle cells that in fact represent a virgin cell population being in a resting not activated state. It should be noted that PAG is relatively strongly expressed and constitutively phosphorylated in resting peripheral blood T-cells [1,3,5,7]. The present observation that PAG is much more strongly expressed in proliferating centrocytes and centroblasts than in resting B-cells could be explained by the hypothesis that Src-family kinases may play somewhat different roles in B-cells than in T-cells. Supposedly, following activation of resting peripheral T-cells, Src-kinases Lck/Fyn phosphorylate immunoreceptor tyrosin-based activation motifs (ITAMs) of signalling subunits of the T-cell receptor, and thus propagate the signal leading, finally to activation of nuclear transcription factors AP1, NFAT and NF κB. In B-cells, however, the phosphorylated Src-kinases may preferentially phosphorylate immunoreceptor tyrosin-based inhibitory motifs (ITIMs) of negative signalling regulators such as CD22 or PIR-B [11]. The latter possibility concurs well with the observation that cross-linking of the B-cell receptor results in phosphorylation of PAG, followed by recruitment of Csk to the membrane rafts [12] resulting in suppression of the Src-kinases that in its effect may temporarily block the negative regulatory signals based on ITIM phosphorylation. Moreover, molecular profiling of a various DLBCL revealed three subgroups of these lymphomas—germinal center B-cell-like, activated B-cell-like, and type 3 DLBCL; four groups of genes predictive of

Table 1
Expression of PAG in various types of lymphoma as determined in the present study

Diagnosis (WHO classification)	Location	Number of cases	PAG
Small lymphocytic lymphoma/chronic lymphocytic leukemia	Lymph node	3	Negative
Mantle cell lymphoma	Lymph node	7	Negative
Extranodal marginal zone lymphoma	Stomach	1	Negative
	Nasopharynx	1	Negative
Splenic marginal zone lymphoma	Spleen	1	Negative
Follicular lymphoma (grade 1–3)	Lymph node	9	Positive
Diffuse large B-cell lymphoma	Lymph node	5	Two positive, three negative

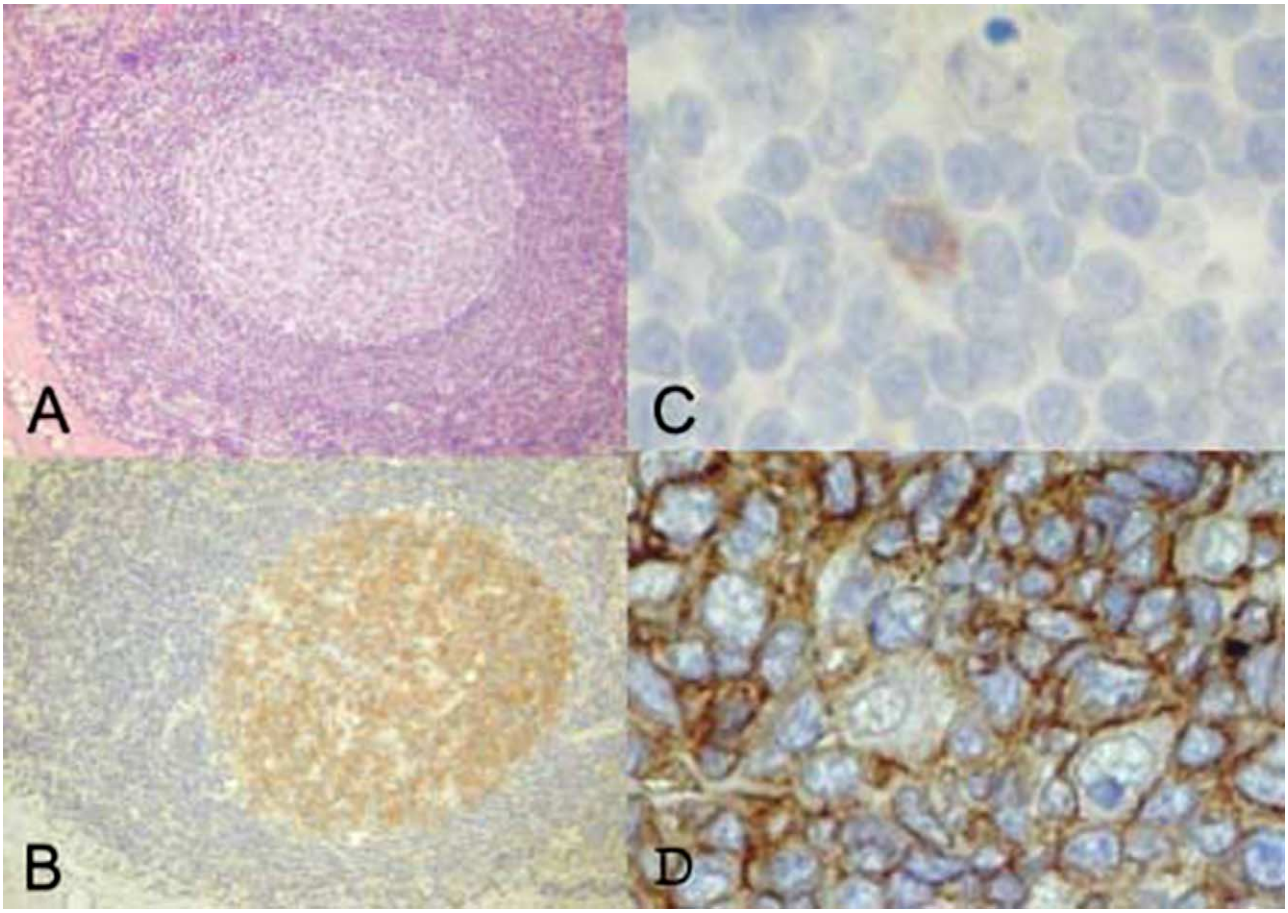

Fig. 1. PAG is relatively specifically expressed in germinal centers of lymph node lymphoid follicle and in follicular lymphoma. Quandrants (A and B): a secondary lymphatic follicle containing a germinal center surrounded by a mantle zone, note strong positivity in the germinal center and negativity in the mantle zone. Quadrant (C): mantle cell lymphoma, note negativity of the lymphoma cells, the single positive cell in the centre is probably a T lymphocyte. Quadrant (D): follicular lymphoma grade 2, note strong positivity of the lymphoma cells. (B–D) Immunostaining with MEM-255 and (A) hematoxylin-eosin staining. Magnification: (A and B) 200× and (C and D) 600×.

chemotherapy were identified [13]. We suspect that the heterogeneous expression of PAG among DLBCL cases might reflect different oncogenic pathways. Further analysis is needed to determine possible correlation of PAG expression with these subtypes and/or chemotherapy response. Recent results on signaling perturbations in lipid rafts of anaplastic large cell lymphoma and Hodgkin lymphoma cell lines involving also PAG [14] lend further support to the idea that PAG may be involved in neoplastic lymphoproliferation. Mechanisms participating in regulation of PAG expression in various subsets and differentiation stages of B lymphocytes remain to be elucidated. Finally, it should be acknowledged that the reactivity of the mAb under the particular conditions used in this study may not perfectly reflect the expression of the PAG molecule. It is imaginable that a critical post-translational modification of a subset of PAG molecules (perhaps differentially occurring in different cell types) may affect the recognition by MEM-255. Nevertheless, based on Western blotting data, MEM-255 reactivity is apparently not compromised by phosphorylation, the major physiological modification of PAG.

From the routine histopathology point of view, PAG (or at least the MEM-255 epitope of PAG) might be a new positive marker of follicular lymphoma and a negative marker of some “small cell lymphomas”.

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ORIGINAL ARTICLE

Expression of transmembrane adaptor protein PAG/Cbp in diffuse large B-cell lymphoma: Immunohistochemical study of 73 cases

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Abstract

PAG/Cbp is a transmembrane adaptor protein involved in proximal immune signaling. It is expressed in reactive germinal centers (GC) of secondary lymphatic follicles and related malignant lymphomas.

We studied PAG/Cbp expression in GC-like and non-GC-like diffuse large B-cell lymphoma (DLBCL) subtypes.

Seventy-three cases of DLBCL identified among 155 malignant lymphomas were classified as GC-like DLBCL (CD10+ or CD10−, bcl-6+, and MUM1−) and non-GC-like DLBCL (CD10−, MUM1+ or CD10−, bcl-6+, MUM1+). PAG/Cbp was detected by monoclonal antibody MEM-255 following routine immunohistochemical procedures.

Thirty-five of 40 GC-like DLBCLs (88%) and 20 of 33 non-GC-like DLBCL cases (61%) expressed PAG/Cbp. Four of 12 bcl-6-negative non-GC-like DLBCL cases (33%) were PAG/Cbp positive, and only 4 of 20 bcl-6-positive non-GC-like DLBCL cases (25%) were PAG/CBP negative. All 37 FL and all 5 Burkitt's lymphomas (BL) expressed PAG/Cbp, whereas all 6 mantle cell lymphomas (MCL) and 4 of 5 chronic lymphocytic leukemias (CLL/SLL) were PAG/Cbp negative.

PAG/Cbp is a reliable GC marker. Its expression correlates with GC-like DLBC phenotype in a significant majority of cases. It is typically absent in MCL and SLL/CLL.

Keywords: PAG/Cbp; Small cell malignant lymphoma; Diffuse large B-cell lymphoma

Introduction

Phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG), also known as C-terminal Src-kinase (Csk) binding protein (Cbp), further referred to as PAG, is a ubiquitously expressed transmembrane adaptor protein anchored in specialized compartments of the plasma membrane called lipid rafts or glycosphingolipid-enriched microdomains [5,14]. Lipid rafts play an important role in antigen trafficking and immune signaling executed by a number of raft-associated signaling molecules, including Src-kinase family proteins (SFK). SFK play a crucial role in the initial phases of signaling processes with complex ramifications involving proliferation, differentiation, secretion, and movement [7,12,30]. Their pivotal role in essential cellular functions requires tight regulation, which is executed particularly by activity of Csk. Csk is a cytoplasmic protein that must be recruited from the cytoplasm to impose its negative regulatory effect on the membrane-bound SFK. This recruitment is executed by phosphorylated PAG [5,14,18].

The following negative regulatory feedback has been recently suggested for T-cells. Upon T-cell receptor

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(TCR) ligation, PAG becomes dephosphorylated as a result of CD45 phosphatase activity. Subsequently, Csk is released from the lipid rafts, and the Csk-mediated suppression of SFK activity is relieved. Finally, SFK, in turn, phosphorylate PAG, which results in re-recruitment of Csk to the lipid rafts. [8,12,26,29] In B-cells, a possible PAG-mediated regulatory effect is more complex and can lead either to stimulation or inhibition depending on the substrate of SFK [10,12].

Experiments investigating the effects of downregulation of PAG expression on bovine peripheral B-lymphocytes suggested its possible role in the pathogenesis of lymphoproliferative disorders [2]. Two recent immunohistochemical studies showed constant PAG expression in FL and a lack of expression in MCL and CLL/SCL. By contrast, PAG expression in diffuse large B-cell lymphoma (DLBCL) was variable [23,25], which prompted us to verify whether PAG could serve as a positive marker of GC-like DLBCL. Herein, we report the results of PAG expression in 73 cases of DLBCL and discuss them along with two other newly reported germinal center (GC) cell markers, HGAL and Jaw1.

Materials and methods

The study group of DLBCL was selected from 155 consecutive cases classified as malignant lymphoma at Hematological Malignancy Diagnostic Service, Leeds General Infirmary, UK, during January, May, and June 2006. Diagnoses were established according to the WHO classification of hematolymphoid malignancies using a standard immunohistochemical panel of antibodies and routine immunohistochemical procedures [13]. A CD10, bcl-6, MUM1 algorithm as published by Hans et al. [11] was used for subclassification of DLBCL into GC-like and non-GC-like types.

PAG was detected by mouse IgG2a monoclonal antibody MEM-255 donated by Prof. Horejsi. It was prepared from a mouse immunized with recombinant human PAG as described elsewhere [5]. The antibody reacts optimally with denatured PAG (under the conditions of Western blotting) irrespective of whether tyrosine phosphorylated or not. The dilution was 1:10, and the immunohistochemical reaction required an antigen retrieval step-heating in citrate buffer, pH 6.0 for 20 min. The Universal LSAB+ Kit (DAKO, Carpinteria, CA) was used according to the manufacturer's recommendation for visualization. MEM-255 reactivity was tested in hyperplastic lymph node tissue (Figs. 1 and 2).

During routine diagnostic procedure, PAG expression was assessed as positive, with uniform strong expression in more than 90% of tumor cells (+), positive in a variable proportion (25–90%) of tumor cells (±), and negative (−). Only membranous positivity was considered as a positive MEM-255 reaction (Figs. 3–5).

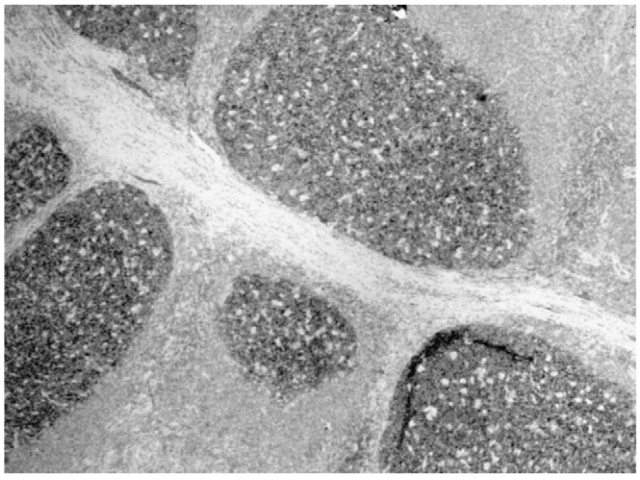

Fig. 1. PAG expression pattern in reactive follicular hyperplasia of the lymph node with strong positivity of the germinal center contrasting with the negative follicle mantle. Antibody MEM-255, magnification 100 × .

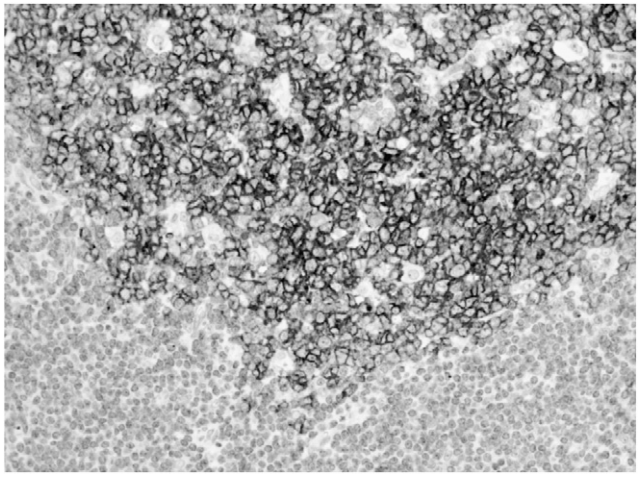

Fig. 2. PAG expression pattern in reactive follicular hyperplasia of the lymph node with strong positivity of the germinal center contrasting with the negative follicle mantle. Antibody MEM-255, magnification 400 × .

Results

The non-neoplastic lymph node tissue with evidence of mixed follicular and paracortical hyperplasia, used as a control, showed a strong positive PAG reaction in GC of secondary lymphatic follicles, clearly contrasting with a negative reaction in the mantle zone, and a considerably weaker positive reaction in the paracortex. Both centrocytes and centroblasts displayed strong membranous positivity, while macrophages remained negative. The reaction pattern concurred with previously published observations [23,25].

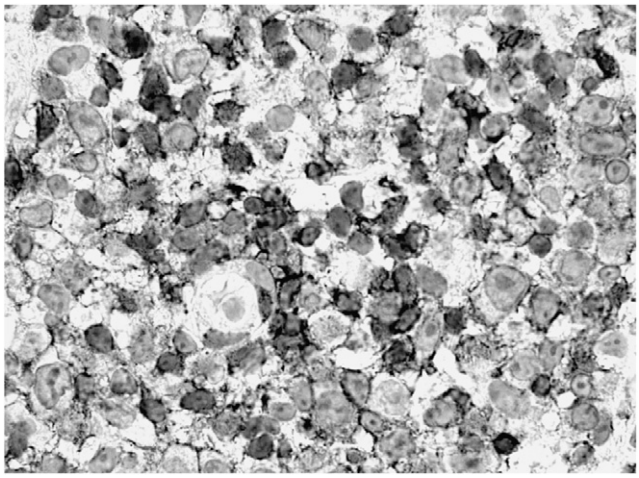

Fig. 3. A representative image demonstrating reaction classified as positive uniform in diffuse large B-cell lymphoma. Antibody MEM-255, magnification 600 × .

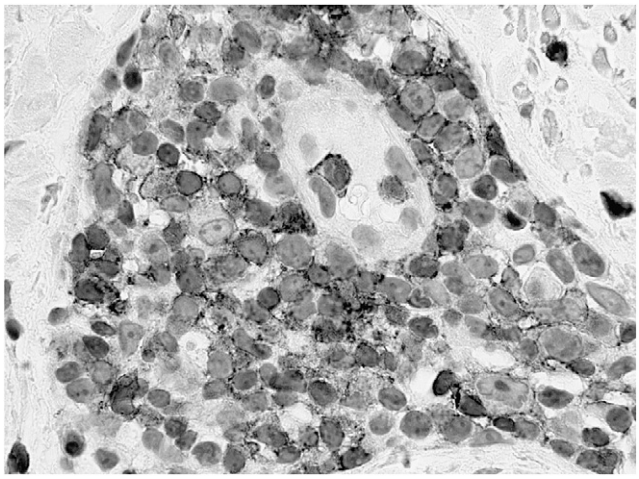

Fig. 4. A representative image demonstrating reaction classified as positive variable in diffuse large B-cell lymphoma. Antibody MEM-255, magnification 600 × .

Among 155 cases of malignant lymphoma, 134 cases were non-Hodgkin B-cell malignant lymphomas, and 73 of these were classified as DLBCL, 37 as FL, 5 as BL, 6 as MCL, and 5 as CLL/SLL. Six cases were T-cell lymphomas represented by two cases in each of the following diagnostic categories: peripheral T-cell lymphoma NOS, anaplastic large cell lymphoma, and precursor T-cell lymphoblastic leukemia. Fifteen cases were Hodgkin lymphomas, 14 of classical type, and 1 nodular lymphocyte predominant Hodgkin lymphoma.

All FL and BL and, unexpectedly, 1 CLL/SLL gave positive reactions, while all MCL and 4 CLL/SLL gave negative reactions. One FL was graded as 3b, and this showed variable positivity. Results are summarized in Table 1.

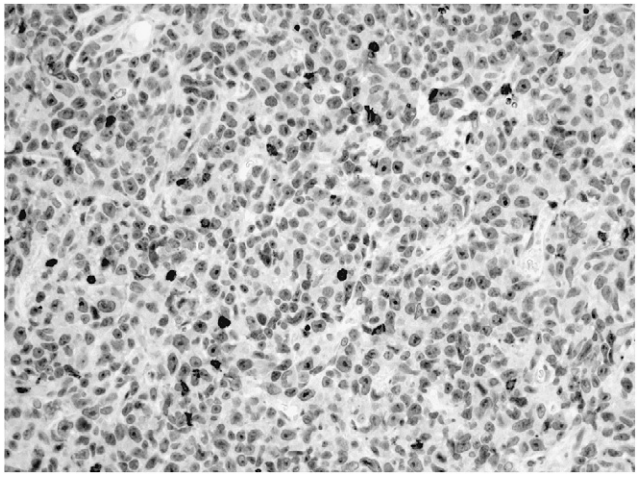

Fig. 5. A representative image demonstrating reaction classified as negative in diffuse large B-cell lymphoma. Antibody MEM-255, magnification 400 × .

Forty DLBCL cases displayed GC-like phenotype – CD10 + (36 cases) or CD10−, bcl-6 +, and MUM1− (4 cases), and 33 DLBCL cases showed non-GC-like phenotype – either CD10−, bcl-6 +, MUM1 + (20 cases) or CD10−, bcl-6-, MUM1 + (12 cases). Bcl-6 status was not determined in one non-GC-like DLBCL expressing immunoprofile CD10− and MUM1 +.

In the GC-like DLBCL group 35 of 40 cases (88%) expressed PAG, all but three strongly. All cases of DLBCL with underlying follicular lymphoma ($n = 11$) expressed PAG, 10 strongly.

In the non-GC-like DLBCL group, 20 of 33 cases (61%) expressed PAG, 17 of them gave strong and 3 variable reactions. Four of 12 bcl-6-negative cases (33%) were PAG positive, and only 4 of 20 bcl-6-positive cases (20%) were PAG negative.

All T-cell lymphomas gave positive reactions. All classical Hodgkin lymphomas did not express PAG in HRS cells, whereas the only case of nodular lymphocyte predominant Hodgkin lymphoma showed a positive reaction in L&H cells. Data are summarized in Table 2.

Discussion

The WHO classification of malignant lymphomas is based on histogenesis and clinical behavior. It is supposed that morphological, immunophenotypic, and genetic characteristics of malignant lymphomas reflect either the stage of development at which malignant transformation occurs or a stage to which the neoplasms have differentiated [13]. The concept makes it possible to use routine histology and immunohistochemistry for malignant lymphoma classification. Although it has long been recognized that the category of DLBCL represents a biologically heterogeneous group of diseases,

Table 1. PAG expression in 155 malignant lymphomas

Diagnosis	Number of cases	PAG+	PAG±	PAG-positive total no.
Chronic lymphocytic leukemia/ small lymphocytic lymphoma	5	0	1	1
Extranodal marginal zone lymphoma	4	0	0	0
Extranodal marginal zone lymphoma with systemic involvement	2	0	0	0
Mantle cell lymphoma	6	0	0	0
Follicular lymphoma (grade 1–3b)	37	35	2	37
Diffuse large B-cell lymphoma	73	49	6	55
Mediastinal large B-cell lymphoma	2	0	0	0
Burkitt's lymphoma	5	5	0	5
Peripheral T-cell lymphoma NOS	2	1	1	2
Anaplastic large cell lymphoma	2	2	0	2
Precursor T-cell lymphoblastic leukemia	2	2	0	2
Classical Hodgkin lymphoma	14	0	0	0
Nodular lymphocyte predominant Hodgkin lymphoma	1	1	0	1
Total	155	95	10	106

Table 2. PAG expression in DLBCL subtypes

DLBCL	PAG+	PAG±	PAG−	Total
GC-like DLBCL	32	3	5	40
Non-GC-like DLBCL – total	17	3	13	33
Non-GC-like DLBCL – bcl-6 positive	14	2	4	20
Non-GC-like DLBCL – bcl-6 negative	3	1	8	12

clinically relevant subclassification was elusive until the era of gene expression profiling [22]. This new molecular biologic technique led to the identification of two biologically different DLBCL subsets, GC-like and non-GC-like (activated-like) DLBCLs, and to the confirmation that the primary mediastinal large B-cell lymphoma is a separate entity [1,19,20,27]. The search for immunohistochemical surrogate markers of gene expression signatures detected by DNA microarrays revealed that a combination of antibodies against CD10, bcl-6, MUM1, and CD138 can discriminate between the GC-like and the non-GC-like DLBCL subtypes [6,11]. Furthermore, recent research identified a new GC cell-associated protein, HGAL, and shed new light on the diagnostic utility of previously characterized proteins, Jaw1 and PAG/Cbp [3,5,14,15]. Data are summarized in Table 3.

All these proteins re-iterate their expression pattern in reactive lymphoid tissue in histogenetically related malignant lymphomas. They are strongly expressed in GC and FL, whereas follicle mantles, MCL, and CLL/SLL give negative reactions. Among DLBCLs, only a proportion of cases show positive reactions, making this heterogeneous category a logical object for further investigation.

HGAL is supposedly a cytoplasmic adaptor protein involved in IL-4-induced pathway [15,16]. It was preferentially expressed in GC-like DLBCs (90% positive GC-like cases versus 52% positive non-GC-like cases) [17]. In addition, it was shown to be associated with a favorable course of the disease [15]. No positive cases of CLL/SLL (8 studied cases) and MCL (7 studied cases) were identified [17]. Interestingly, recent experiments with HGAL-deficient knock-out mice have shown that the analogous mice protein M17 is not essential for the GC reaction in mice [21].

Jaw1 protein is an integral transmembrane protein of the endoplasmic reticulum of lymphoid cells, involved in protein trafficking [3,4]. A recent immunohistochemical study detected this protein not only in 72% of the GC-like DLBCL, but also in 46% of non-GC-like DLBCL [24]. Furthermore, the positive reaction was observed in 13 of 15 cases of CLL/SLL (positivity restricted to proliferation centers was noted in 8 of those cases) and in 2 of 18 MCL.

PAG is a ubiquitously expressed transmembrane adaptor protein involved in early stages of immune signaling setting a threshold on T-cell activation by recruitment of Csk, a major regulator of SFK, to lipid rafts. Its role in B-cells, however, varies due to different effects that SFK might induce. Consequently, PAG can play a part either in negative or positive regulatory loops [10,12]. Similarly to HGAL, two recent experiments with PAG-deficient knock-out mice and PAG gene-disrupted mice showed that PAG is not essential either as a T-cell activity gatekeeper or as a factor essential for thymic development and embryogenesis [9,28]. Despite uncertainty about the role of PAG in T-cell development, PAG still deserves attention, as it is easily and

Table 3. Comparison of PAG, HGAL, and Jaw1 expression in some malignant lymphomas

Antibody/reference diagnosis	PAG, present study	PAG, Ref. [25]	HGAL, Ref. [17]	HGAL, Ref. [24]	Jaw 1, Ref. [24]
SLL/CLL	1/5	0/19	0/8	nd	13/15
Mantle cell lymphoma	0/6	1/19	0/7	nd	2/18
FL grade 1, 2, 3	37/37	90/105	103/107	nd	83/106
DLBCL GC-like	35/40	28/32	61/68	29/32	24/32
DLBCL non-GC-like	20/33	7/30	36/68	12/28	13/28

nd – not done, number of positive cases/total number of cases.

reliably identifiable by routine immunohistochemistry, and so far only three studies have been published addressing its expression pattern in human tissues, with no study investigating its prognostic potential [23,25].

We present results of PAG expression detected by routine immunohistochemistry in a group of 73 DLBCL cases. We observed that PAG was expressed in a majority of GC-like-DLBCL cases defined by the CD10, bcl-6, MUM1 algorithm, including all 11 DLBCL cases with underlying follicular lymphoma. PAG was also expressed in a significant proportion (61%) of non-GC-like DLBCL cases, particularly in those expressing bcl-6. The invariable expression of PAG in FL, a continuous decrease in expression, and finally a complete loss of expression in non-GC-like DLBCL along with a loss of GC marker bcl-6 indicate gradual disintegration of proximal signaling complexes suggestive of increasing independence on survival signals from local microenvironment and consequently a more aggressive course of the disease. However, the prospective character of the study and a small number of non-GC-like cases prevents further analysis of prognostic implications of PAG expression.

Eighty-five percent of all bcl-6-positive cases in the present study were also PAG positive. According to the published data, HGAL and Jaw1, proposed markers of GC differentiation, also show association with bcl-6 expression. Co-expression of PAG, HGAL, and Jaw1 proteins with bcl-6 was observed in 73%, 90%, and 71% of all bcl-6-positive cases, respectively, and hierarchical cluster analysis of immunohistochemical expression of HGAL, CD10, bcl-6, MUM1, and bcl-2 showed clustering of HGAL with bcl-6 and CD10 in one branch of the dendrogram, while MUM1 and bcl-2 clustered in the other branch [17,24,25]. It remains to be investigated whether expression of some or all of these newly identified GC-associated proteins in non-GC-like DLBCL defined by Hans' criteria [11] can have a prognostic impact.

The difference in PAG expression among GC-like and non-GC-like DLBCL in the present study was less marked than in the previous study documenting positive PAG reaction in 88% of 32 GC-like DLBCLs and 23% of 30 non-GC-like DLBCLs [25]. The reason probably lies in the evaluation criteria, and it is possible that some of the cases we considered positive would be classified as negative by the authors. Their evaluation criteria were not shown, and the published image would be classified as a strong reaction according to our criteria.

As for other lymphoma types, our findings are in accordance with the reported constant PAG expression in FL and a lack of expression in MCL and CLL/SLL. Interestingly, we encountered one positive case of CLL/SLL. At present, there are data available on 32 cases of MCL, of which only one was positive. Similarly, only one of 27 cases of CLL/SLL expressed PAG [23,25, present study]. The results suggest that PAG might serve as an adjunct in the differential diagnosis of small cell malignant lymphomas.

In summary, our observations indicate that PAG is a reliable marker of GC differentiation, identifying a slightly broader range of B-cells than conventional GC markers CD10 and bcl-6. It is expressed in a significant majority of GC-like DLBCL cases. It is typically absent in MCL and SLL/CLL. Its significance in terms of prognosis needs to be evaluated.

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4. Conclusions

- Monoclonal antibody MEM 255 detects PAG reproducibly in paraffin embedded lymphoid tissue under routine conditions.
- PAG is a reliable positive marker of germinal centre cells and a negative marker of mantle zone cells.
- PAG can serve as an adjunct in the differential diagnosis of ‘small cell malignant lymphomas’. It is strongly and consistently expressed in follicular lymphoma while it is constantly lacking in mantle cell lymphoma and chronic lymphocytic leukaemia/small lymphocytic lymphoma.
- PAG expression in diffuse large B-cell lymphoma indicates the germinal centre-like phenotype. PAG is expressed in a majority of germinal centre-like diffuse large B-cell lymphomas and in a minority of non-GC-like diffuse large B-cell lymphomas if classified according to the CD10, bcl-6, MUM1 algorithm.
- PAG expression in malignant lymphomas is a sign of preserved B-cell receptor-mediated immune signalling which may imply a certain dependence on environmental factors, less growth autonomy and better outcome. However, significance of PAG as a prognostic marker remains to be investigated.
- PAG can serve as an adjunct in the differential diagnosis between classical Hodgkin lymphoma and nodular lymphocyte predominant Hodgkin lymphoma. A positive reaction lends strong support to the nodular lymphocyte predominant Hodgkin lymphoma. Verification in a larger series of cases is necessary.
- PAG is weakly expressed in T-zones of hyperplastic lymphatic tissue and in some T-cell malignant lymphomas.

5. Summary

5.1. Summary in English language

Introduction: New notions about cell development, cell cycle, differentiation and cell signalling are now being used for refinement of classification of malignant lymphomas (ML) and for development of new drugs specifically targeting deregulated proteins some of which play a role in immune signalling.

The first step in most of the signalling pathways is transduction of the signal through the plasma membrane mediated by a plasma cell receptor. Certain proximal signalling molecules such as a family of Src-kinases (SFK) are segregated in particular plasma membrane compartments called lipid rafts or glycosphingolipid-enriched microdomains (GEM).

SFK are a family of phosphokinases involved in early signalling events. Their deregulation can result in neoplastic transformation.

In lymphoid cells, SFK are tightly regulated by C-terminal Src-kinase which needs to be recruited to the plasma membrane from cytoplasm to execute its regulatory effect. This is mediated by phosphoprotein associated with GEM (PAG) or Csk-binding protein.

PAG is a ubiquitously expressed 80-kDa transmembrane adaptor protein anchored in GEM. In resting T-cells a phospho-PAG/Csk complex suppresses activity of SFK. Following T-cell receptor ligation, PAG becomes dephosphorylated as a result of CD45 phosphatase activity. Subsequently, Csk is released from the lipid rafts and the Csk-mediated suppression of SFK activity is relieved. Finally, SFK in turn phosphorylate PAG resulting in re-recruitment of Csk to the membrane rafts. A PAG-mediated regulatory effect in B-cell immune signalling is more complex and not fully elucidated at present.

The experiment investigating effects of down-regulation of PAG expression on bovine peripheral B-lymphocytes suggested its possible role in pathogenesis of lymphoproliferative disorders.

Moreover, PAG appears necessary for Rituximab, an anti-CD 20 antibody-based drug used in therapy of malignant lymphomas (ML), to impose its toxic effect *in vitro*.

Some of ML entities delineated in the recent WHO classification are heterogeneous if studied at the gene expression level. This heterogeneity correlates with a course of the disease and responsiveness to therapy. This correlation could not be found if the tumours were investigated with conventional morphologic methods.

Diffuse large B-cell lymphoma (DLBCL) an umbrella entity for at least two distinct neoplasms detected by gene expression profiling which are the germinal centre (GC)–like and the non-germinal centre (non-GC)-like DLBCL. Some studies indicated that the former can be recognized immunohistochemically by expression of GC cell-associated molecules CD10 and bcl-6 while the latter by post-GC cell associated molecules such as MUM1.

I studied expression of PAG in non-neoplastic and neoplastic lymphoid tissue in order to determine its expression pattern and to investigate its utility in routine diagnostic haematopathology.

Material and Methods: PAG was detected in lymph node excision biopsies submitted for routine histopathological examination by mouse IgG2a monoclonal antibody MEM-255 donated by Prof. Horejsi, UMG, CAS, Praha. The Universal LSAB+ kit (DAKO, Carpinteria, CA) was used according to the manufacturer's recommendation to visualise the reaction.

Results: A small pilot study showed that the germinal centres of secondary lymphatic follicles were strongly PAG positive whereas small lymphocytes of follicle mantles were negative. The paracortical T-cell area displayed a weak positive reaction. Among ML, follicular lymphomas (FL) were strongly positive (9/9). By contrast, mantle cell lymphomas (MCL)(7/7), chronic lymphocytic leukaemia/small lymphocytic lymphomas (CLL/SLL) (3/3), extranodal marginal zone lymphomas (MZL) (3/3) and plasmacellular neoplasms (2/2) were negative. DLBCL were heterogeneous with 2 positive and 3 negative cases. Classical Hodgkin lymphomas (7/7) gave negative reactions.

Results in the subsequent larger study were as follows: 35 of 40 GC-like DLBCLs (88%) and 20 of 33 non-GC-like DLBCL cases (61%) expressed PAG. All 37 FL and all 5 Burkitt's lymphomas expressed PAG whereas all 6 MCL and 4 of 5 SLL/CLL were PAG negative.

Conclusions: Monoclonal antibody MEM 255 detects PAG reproducibly in paraffin embedded lymphoid tissue under routine conditions. PAG is strongly and consistently expressed in the germinal centres of secondary lymphatic follicles while the follicle mantles remain negative. Correspondingly, germinal centre cell derived ML such as FL and a significant proportion of GC-like DLBCL detected by the CD10,bcl-6 and MUM1

algorithm give positive reactions whereas some other 'small cell lymphomas' such as MCL and CLL/SLL and a considerable proportion of non-GC-like DLBCL give negative reactions. Moreover, HRS cells of classical Hodgkin lymphoma lack PAG expression whereas L&H cells of NLPHL are PAG positive. In summary, PAG can be used as an adjunct in subtyping of DLBCL and in the differential diagnosis of 'small cell lymphomas' and Hodgkin lymphoma.

5.2 Souhrn v českém jazyce

Úvod: Nové poznatky o buněčném vývoji, regulacích buněčného cyklu a signalizaci jsou nyní využívány pro zpřesnění klasifikace maligních lymfomů a pro vývoj nových léků s cíleným efektem na patologicky deregulované molekuly, z nichž některé hrají roli v procesu imunitní signalizace.

První krok buněčných signálních kaskád je většinou přenos signálu přes buněčnou membránu zprostředkovaný mebránovým receptorem. Některé signální molekuly jsou segregovány do speciálních kompartmentů buněčné membrány, tzv. rafts, nebo glycosphingolipid-enriched microdomains (GEM), někdy označovaných jako signalosomy. Mezi tyto molekuly patří i rodina Src-kináz, která reprezentuje skupinu fosfokináz účastnících se časných fází imunitní signalizace. Jejich deregulace může vést k nádorové transformaci buňky.

SFK lymfoidních buněk jsou přísně regulovány C-terminal Src-kinázou, která pro uplatnění svého regulačního vlivu musí být nejprve translokována do cytoplazmatické membrány. Translokace je zprostředkována proteinem phosphoprotein-associated with GEM (PAG), neboli Csk-binding proteinem.

PAG je protein o molekulové hmotnosti 80-kDa zakotvený v GEM; je exprimován v řadě tkání. U T-buněk v klidovém stavu fosforylovaný PAG/Csk komplex potlačuje aktivitu SFK. Po obsazení T-receptoru je PAG defosforylován aktivitou fosfokinázy CD45. Následně je Csk uvolněna z GEM, což vede ke znovuobnovení aktivity SFK, které fosforylují PAG, což má za následek translokaci Csk do GEM. Regulační vliv PAG na signalizaci B-buněk je složitější a v současné době není zcela objasněn.

Experiment zkoumající efekt snížené exprese PAG na bovinní periferní B-lymfocyty ukázal na jeho možnou roli v patogenezi lymfoproliferativních onemocnění.

Podle *in vitro* studie se PAG zdá být nepostradatelným pro funkci Rituximabu, což je lék založený anti-CD20 protilátkové aktivitě používaný v terapii maligních lymfomů (ML).

Některé nozologické jednotky vymezené v současné klasifikaci maligních lymfomů jsou

na úrovni genové exprese heterogenní, tato heterogenita koreluje s heterogenitou jejich klinického průběhu onemocnění a odpovídavostí na léčbu. Tato korelace nebyla zřejmá při zkoumání konvenčními morfologickými metodami.

Difúzní velkobuněčný lymfom z B-buněk (DLBCL) zahrnuje nejméně dvě jednotky detegované metodou známou jako gene expression profiling - germinal centre (GC)–like a non-germinal centre (non-GC)-like DLBCL. Některé studie ukázaly, že ´gene expression profile´ GC-like DLBCL koreluje s expresí markerů exprimovaných buňkami zárodečného centra, jako jsou CD10 a bcl-6, zatímco ´gene expression profile´ non-GC-like DLBCL koreluje s expresí molekul post-germinální fáze vývoje lymfocytu jako je MUM1.

Ve své studii jsem se věnoval expresi PAG v nenádorové a nádorové lymfoidní tkáni a jejímu možnému využití v rutinní diagnostické hematopatologii.

Materiál a metody: PAG byl detegován ve tkáni lymfatických uzlin získaných bioptickou excizí a zaslaných k histologickému vyšetření. K detekci byla použita protilátka MEM-255 darovaná prof. V.Hořejším z ÚMG AV ČR v Praze. Reakce byla vizualizována kitem Universal LSAB+ (DAKO, Carpinteria, CA) aplikovaným podle pokynů výrobce.

Výsledky: Malá pilotní studie ukázala, že zárodečná centra sekundárních lymfatických folikulů jsou silně pozitivní, zatímco lymfoidní buňky pláště jsou negativní. Parakortikální T-oblasti dávaly slabě pozitivní reakci. Folikulární lymfomy (FL) byly silně pozitivní (9/9). Naproti tomu lymfomy plášťové zóny (MCL) (7/7), chronické lymfatické leukémie/lymfomu malých lymfocytů (CLL/SLL) (3/3), extranodální lymfomy marginální zóny (MZL) (3/3) a nádory z plazmatických buněk (2/2) byly negativní. DLBCL byly heterogenní se 2 pozitivními a 3 negativními případy. Klasický Hodgkinův lymfom (7/7) dával negativní reakce.

Výsledky v následné studii zahrnující větší počet případů ukázaly: 35 ze 40 GC-like DLBCLs (88%) a 20 ze 33 non-GC-like DLBCL (61%) exprimovaly PAG. Všech 37 FL a všech 5 Burkittových lymfomů exprimovalo PAG, zatímco všech 6 MCL a 4 z 5 CLL/SLL byly PAG negativní.

Závěry: Monoklonální protilátka MEM-255 deteguje spolehlivě a reprodukovatelně PAG v lymfoidní tkáni. PAG je silně pozitivní v zárodečných centrech sekundárních lymfatických folikulů, zatímco reakce v plášťových zonách je negativní.

Maligní lymfomy odvozené z buněk zárodečných centrech jako FL a významná část GC-like DLBCL diagnostikovaná pomocí algoritmu CD10, bcl6 a MUM1 je rovněž pozitivní na rozdíl od tzv.lymfomů z malých buněk jako jsou MCL a CLL/SLL. HRS buňky klasického Hodgkinova lymfomu PAG neexprimují, zatímco L&H buňky nodulárního Hodgkinova lymfomu s lymfocytární predominancí jsou PAG pozitivní.
Výsledky studie svědčí pro to, že PAG lze využít jako pomocný marker při subtypizaci difúzního velkobuněčného lymfomu z B-buněk, diferenciální diagnóze 'lymfomů z malých buněk' a diferenciální diagnóze Hodgkinova lymfomu.

6. List of author´s publications

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