Absence of Immunoglobulin Class Switch in Primary Lymphomas of the Central Nervous System

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Primary lymphomas of the central nervous system (PCNSLs) were investigated for their capacity to perform further maturation steps. We studied a series of 11 PCNSLs derived from immunocompetent patients for immunoglobulin (Ig) class switch recombination (CSR) by performing reverse transcriptase-polymerase chain reaction (RT-PCR) for transcripts of Ig constant region gene segments (IGHC). This analysis revealed exclusive transcription of IgM and IgD mRNA in the absence of IgG, IgA, or IgE transcription. This finding was corroborated at the protein level by the immunohistochemical demonstration of IgM on the surface of the tumor cells. The unexpected lack of CSR may be due to internal switch μ region deletions, which were detected in 7 of 11 cases. We also found that expression of activation-induced cytidine deaminase (AID), which is required for CSR and somatic hypermutation, was detectable by RT-PCR in 4 of 10 cases and by immunohistochemistry in one of three cases analyzed. This may indicate that ongoing somatic mutation, which is often observed in PCNSL, could be due to sustained AID expression in a fraction of cases and that intraclonal V gene diversity may occur in other cases at an earlier phase of tumor clone expansion, when AID may have been expressed. (*Am J Pathol* 2005, 166:1773–1779)

Primary central nervous system lymphomas (PCNSLs) are highly malignant non-Hodgkin's lymphomas of the diffuse large B-cell type (DLBCL) with a poor prognosis.^{1–3} Molecular analysis of their immunoglobulin (Ig) genes identified germinal center (GC) B cells as their cellular origin.^{4,5} PCNSLs are characterized by the introduction of somatic mutations into their rearranged Ig genes at high frequencies. Remarkably, their mean mutation frequencies for the Ig heavy and light chain V region genes reach 13.2 and 8.3%, respectively, thereby exceeding other GC B-cell-derived lymphoma entities as well as normal, nonmalignant post-GC B cells.^{6,7} These data suggest a prolonged participation of the tumor cells or their precursor cells in a GC reaction. Furthermore, there is evidence for ongoing somatic V gene mutation during tumor clone expansion.^{4,5,8}

These observations raise the question of whether the tumor cells of PCNSL may perform further maturation steps, including Ig class switch recombination (CSR). CSR replaces the μ constant region with one of the downstream-located constant regions, allowing the generation of different antibody classes.⁹ CSR occurs within the 3- to 5-kb repetitive switch region sequences located 5' of each constant region gene segment (IGHC). Although the precise molecular mechanisms of CSR have not yet been fully elucidated, the essential and sole (human) B-cell-specific known factor required for CSR is the enzyme activation-induced cytidine deaminase (AID).^{10,11}

In AID-deficient mice, both CSR and somatic hypermutation (SHM) are abolished, illustrating that this protein plays a pivotal role in both processes, thus, closely linking SHM and CSR.¹² The pattern, distribution, and levels of AID protein in various B-cell subsets and B-cell neoplasms have only incompletely been determined. RNA transcription analysis indicated a selective expression of AID in GC B cells and in follicular lymphoma and DLBCL,

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which are GC B-cell-derived lymphomas.^{11,13,14} However, the expression of AID in PCNSL has not yet been analyzed. The aim of the present study was to clarify whether tumor cells of PCNSL had undergone CSR and to assess AID mRNA and protein levels in this CNS-specific subtype of DLBCL.

Materials and Methods

Histopathology

Stereotactic tumor biopsies of 11 immunocompetent patients (five female and six male; mean age, 62; range, 28 to 75 years) with a histopathologically confirmed diagnosis of PCNSL were analyzed. All studies were approved by local Ethics Committees; informed consent was provided according to the Declaration of Helsinki. Systemic lymphoma manifestation was excluded by extensive staging. Human nonmalignant tonsils were obtained with written consent from patients undergoing tonsillectomy. All tumors were histopathologically classified as DLBCL according to the World Health Organization classification.² The diagnoses were based on a combination of routine morphology and immunohistochemistry with antibodies against Ki-67 (clone MIB-1; Dako, Hamburg, Germany), CD45 (clone T29/33; Dako), CD3 (polyclonal; Dako), and CD20 (clone L26; Dako), as reported before.⁴ In addition, immunohistochemical analyses were performed with monoclonal mouse antibodies against IgM (clone R1/69; Dako) and IgG (clone A57H; Dako). An ABC protocol was applied using 3,3'-diaminobenzidine (Sigma, Deisenhofen, Germany) as chromogene and H₂O₂ as co-substrate. Expression of AID was investigated in three PCNSLs (cases 6, 10, and 11) using the monoclonal rat anti-human AID antibody EK2-5G9 (IgG_{2b}).¹⁵ Briefly, frozen sections were fixed in 4% paraformaldehyde and subjected to antigen retrieval as described previously¹⁵ before application of the primary antibody. Bound antibody was detected using an APAAP protocol with rabbit anti-rat IgG and rat APAAP (both from Dakocytomation, Hamburg, Germany).

Isolation of GC B Cells

Human tonsils were minced and mononuclear cells were obtained through a Ficoll density gradient (Amersham Biosciences, Freiburg, Germany). B cells were enriched with CD19-coupled magnetic beads (Miltenyi, Bergisch-Gladbach, Germany). Thereafter, GC B cells were stained with monoclonal mouse anti-human CD38-PE (BD, Heidelberg, Germany) and CD77-FITC (BD). Centrocytes and centroblasts were sorted as CD38⁺CD77⁻ and CD38⁺CD77⁺ cells, respectively, using a FACS Vantage cell sorter (BD).

DNA Extraction

DNA was extracted from frozen tissue blocks harboring at least 80% tumor cells with the NucleoSpin Tissue kit (BD Clontech, Heidelberg, Germany). DNA was dis-

solved in 100 μ l of TE buffer (pH 7.6). Two to 5 μ l of this stock DNA solution, corresponding to 100 ng of DNA, was used in each amplification reaction.

RNA Extraction

RNA was extracted from frozen tumor tissue blocks, which contained at least 80% tumor cells, either with the SNAP total RNA isolation kit (Invitrogen, Karlsruhe, Germany) or using the trizol/chloroform method. First-strand cDNA was either synthesized using the SuperScript II kit (Invitrogen) or using the High Capacity cDNA Archive kit (Applied Biosystems, Weiterstadt, Germany).

Polymerase Chain Reaction (PCR) Analysis

Rearrangements of the immunoglobulin heavy (IgH) chain locus were amplified with VH gene segment family-specific primers from genomic DNA as published previously.⁴ Ten microliters of each PCR reaction was analyzed on a 2% agarose gel stained with ethidium-bromide. PCR products were extracted from a 2% low-melting agarose gel (NuSieve GTG Agarose; Biozym, Hessisch-Oldendorf, Germany) using the QIAEx II kit (Qiagen, Hilden, Germany) and directly sequenced from both sides using the BigDye kit (Applied Biosystems) and an ABI377 automated sequencer (Applied Biosystems). Sequences were compared with human germline Ig gene sequences using the IMGT¹⁶ and GenBank databases and DNAPlot, MacVector, and Blast software.

The switch μ ($S\mu$) region was amplified using IgMrev, 5'-TCG TAT CCG ACG GGG AAT TCT CAC AG-3', and $S\mu$ for, 5'-TCC ATC CAG CTT TCA GAA ATG GAC TC-3', as reverse and forward primers, respectively, applying the GeneAmp XL PCR system (Applied Biosystems) supplemented with 0.75 mmol/L Mg(OAc)₂ and 1.5 $\times$ PCR Enhancer solution (Invitrogen). Cycling conditions were 95°C for 5 minutes, 44 cycles at 95°C for 60 seconds, 62°C for 45 seconds, and 72°C for 6 minutes, followed by a final extension step at 72°C for 15 minutes. Two PCR products were isolated from agarose gels and directly sequenced from both sites using the BigDye kit.

Reverse Transcriptase (RT)-PCR Analysis

All cDNAs were tested by RT-PCR for transcripts of β -actin as housekeeping gene (accession no. nm_001101). As primers, BA-F, 5'-TTT CTT GAC AAA ACC TAA CTT GCG-3', and BA-R, 5'-TAG GAT GGC AAG GGA CTT CTT G-3', were used, amplifying a 266-bp product (position 1231–1496). Amplification was carried out for one cycle at 95°C for 5 minutes, 60°C for 30 seconds, and 72°C for 1 minute, followed by 24 cycles at 95°C for 50 seconds, 60°C for 30 seconds, and 72°C for 1 minute. A final extension step at 72°C for 10 minutes was added. Standard TaqDNA polymerase (Invitrogen) was added before PCR. PCR reactions without DNA served as negative control.

Lymphoma-derived cDNAs were tested by PCR for transcripts of IGHC gene segments. A clone-specific

Table 1. Immunoglobulin Isotype Expression in PCNSL

Patients	IGHV	Mut (%)	CDR3 primer (5' → 3')	Isotype of tumor cells
1	V3-7	8.9	GAATGCGGGGAGTAATAATC	IgM+IgD
2	V3-74	12.2	GAGAATATAACGTCTAAACC	IgM+IgD
3	V3-23	22.2	AAGACGACGGCAAAGGTGGTG	IgM+IgD
4	V3-7	12.6	CGTGGATCGGGAAGTGACGAC	IgM+IgD
5	V4-34	15.5	CTATACGGTAGTGACTCCGAAG	IgM+IgD
6	V4-34	4.1	CCACTGCCAGCTGGTATGATG	IgM+IgD
7	V4-59	21.0	AGTGCGACCTATGGCCGGCTC	IgM+IgD
8	V4-34	23.2	CATGAACGTCTCTGAACGGC	IgM+IgD
9	V4-34	6.9	GATTCGCTCCGGGTATAGCAG	IgM+IgD
10	V3-48	8.2	CGGGCGATGGCCGCTACGGCA	IgM+IgD
11	V4-34	18.4	CGAGTAGTCGACGACGACTCC	IgM+IgD

Mut, mutation frequency of the IGHV compared with the most homologous germline gene segment. Patients 1 and 3 have been included in a previous publication⁴ corresponding to patients 9 and 3, respectively.

forward primer, corresponding to a fragment of the complementarity-determining region 3 (CDR3; Table 1), was used together with an isotype-specific reverse primer in five different PCRs. For isotype IgM: IGHC-M, 5'-GTG GGA CGA AGA CGC TCA CTT TGG-3'; for isotype IgD: IGHC-D, 5'-CTG GCC AGC GGA AGA TCT CCT TC-3'; for isotype IgG1, IgG2, IgG3, and IgG4: IGHC-G, 5'-CTT GTC CAC CTT GGT GTT GCT GG-3'; for isotype IgE1 and IgE2: IGHC-E, 5'-GAC GAC TGT AAG ATC TTC ACG GTG-3'; and for isotype IgA1 and IgA2: IGHC-A, 5'-ACA GTC ACA TCC TGG CTG GRA TTC-3' were applied, respectively. Amplification was carried out for one cycle at 95°C for 3 minutes, 57°C for 30 seconds, and 72°C for 1 minute, followed by 34 cycles at 95°C for 30 seconds, 57°C for 30 seconds, and 72°C for 1 minute. A final extension step at 72°C for 10 minutes was added. Platinum *Taq*DNA polymerase (Invitrogen) was added before PCR. As positive controls, cDNAs derived from human tonsils and various Epstein-Barr virus-transformed B-cell lines (J8 and J17) were used; as negative controls, HeLa cell line-derived cDNA and water instead of cDNA were used, respectively.

AID mRNA expression was detected by quantitative real-time PCR using the Applied Biosystems Assays-on-Demand Gene Expression Product on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). As positive controls, cDNAs derived from normal human purified tonsillar centroblasts and centrocytes, Raji B cells, and different Epstein-Barr virus-transformed B-cell lines were used. As negative controls, we selected HeLa cell line-derived cDNA and water instead of cDNA. As endogenous control genes, the TATA box binding protein and RNA polymerase II were used. All samples were studied in triplicate. Analyses were performed according to the $\Delta\Delta$ CT method. AID mRNA expression in the various samples was expressed relative to AID mRNA expression in centroblasts.

Results

The data for IgH rearrangements detected in the 11 PCNSLs analyzed are summarized in Table 1. All tumors carried potentially functional immunoglobulin heavy chain variable gene segment (IGHV) gene rearrange-

ments. Forty-five percent (5 of 11) of the tumors used an IGHV of the VH3 gene family, whereas the remaining 55% (6 of 11) rearranged a gene segment of the VH4 gene family (Table 1), with five of these six PCNSLs having rearranged the IGHV4-34 gene segment. This preferential usage of the V4-34 gene segment in PCNSL confirms earlier data.^{4,5} The mean mutation frequency of this series was 13.9%, with a range from 4.1 to 23.2%. All rearrangements were in-frame, and stop-codons were not identified. Thus, all rearrangements were considered as potentially functional. Sequences of the IgH rearrangements derived from patients 1 to 11 are available through GenBank accession nos. AF168818 and AY598953 to AY598962.

To determine the Ig isotype of the tumor cells, individual clone-specific primers, localizing to the CDR3, were designed for each tumor and used together with primers specific for the various constant region genes. RT-PCR detected IgM and IgD mRNA in all tumors, whereas no IgG, IgA, or IgE mRNA transcripts were expressed in any of the 11 PCNSLs (Table 1). This observation was confirmed and extended at the protein level: immunohistochemistry revealed IgM expression on the surface of the tumor cells in all PCNSLs (Figure 1, a and b), whereas IgG protein was consistently absent from the malignant cells (Figure 1, c and d).

Because AID is required for both SHM and CSR, AID mRNA and protein expression were further investigated in 10 and 3 PCNSLs, respectively. Real time PCR detected transcription of AID mRNA levels comparable with centroblasts and centrocytes in three PCNSLs, whereas AID mRNA was virtually absent from six PCNSLs. AID mRNA levels in only one PCNSL (case 6), which was derived from a cerebellar tumor of a 60-year-old patient, exceeded the levels detected in centroblasts and centrocytes (Figure 2). These mRNA data were confirmed at the protein level. In the latter patient, a significant fraction of the tumor cells expressed AID (Figure 1e), whereas AID protein was absent from two further PCNSLs (Figure 1f, cases 10 and 11), which also did not transcribe AID mRNA (Figure 2).

Based on the observation that some human IgM-expressing B cells carry internal deletions of the $S\mu$ region, it has been speculated that these deletions may impair

Figure 1. Immunohistochemical phenotype of PCNSL. **a** and **b**: Strong IgM expression on the surface of the tumor cells in a PCNSL (**a**) as well as in the GC of a tonsil (**b**). Anti-IgM immunostaining, slight counterstaining with hemalum, $\times 200$. **c** and **d**: Tumor cells do not express IgG (**c**), whereas IgG staining is visible in a tonsillar GC (**d**). Anti-IgG immunostaining, slight counterstaining with hemalum, $\times 200$. **e** and **f**: AID can be detected in some tumor cells of a cerebellar tumor (**e**) but is not expressed in PCNSL in characteristic supratentorial location (**f**), $\times 100$. **Inset I**: negative control with omission of the primary antibody; **inset II**: positive control with AID expression in the GC of a tonsil, $\times 100$.

class switch recombination to downstream IgH genes and hence serve as a means of isotype stabilization.¹⁷ To find out whether the consistent absence of class switching in PCNSL may be associated with such internal $S\mu$ deletions, we performed a long-distance PCR to amplify the $S\mu$ region from the PCNSL. In all 11 cases, the full-length PCR product of 4.5 kb was obtained, confirming

that the DNA was of sufficient quality to amplify the wild-type $S\mu$ region. In 6 of 11 cases, one shorter PCR product was obtained, and in 1 case, two shorter PCR products were obtained, in addition to the full-length amplicate (Figure 3A; data not shown). All of these shorter products were confirmed in independent PCR reactions. Moreover, DNA from the Jurkat T cell line used as control never

Figure 2. AID mRNA transcription in PCNSL. Quantitative RT-PCR analysis of AID normalized to RNA polymerase II expression and relative to centroblasts (CB). In PCNSL (open bars), AID mRNA levels are generally low compared with centroblasts (CB) and centrocytes (CC). Only one PCNSL (no. 4), which was located in the cerebellum, transcribed increased levels of AID mRNA. B, Raji cell line-derived cDNA; H, HeLa cell line-derived cDNA. The $\Delta\Delta CT$ method was used. Normalization to TATA box binding protein yielded identical results (data not shown). Samples were analyzed in triplicate.

gave a shortened PCR product in any of 53 PCR reactions, further ruling out that the shortened products were due to amplification artifacts of the highly repetitive $S\mu$ region. Finally, in two cases with sufficient amounts of PCR product, the amplicates were directly sequenced, confirming that they in fact represent $S\mu$ regions with internal deletions (Figure 3B). Thus, at least a large fraction of PCNSLs carry internal $S\mu$ deletions. Due to lack of further tumor material, it was not possible to clarify whether in the six cases with one deleted $S\mu$ region the deletion was present on the IgH allele expressed and whether the full-length product was derived from nontumor cells in the tissue sample (as is likely the case for the PCNSL with two deleted $S\mu$ regions) or from the second allele of the PCNSL clone.

Discussion

This study demonstrates that PCNSLs have not switched their Ig isotype, a process that commonly follows onset of SHM in the GC, indicating that the tumor cells, which originate from GC B cells,⁴ did not perform further steps of maturation.

Lack of CSR was documented by both RT-PCR and immunohistochemistry, demonstrating an IgM/IgD phenotype and an expression of IgM on the surface of the tumor cells in all 11 PCNSLs of this series. Remarkably, AID mRNA, a prerequisite for CSR, was expressed at levels comparable with or even exceeding levels detected in normal GC B cells in 4 of 10 cases analyzed. Immunohistochemistry in three cases paralleled the quantitative RT-PCR results, showing absence of AID protein in two RT-PCR-negative cases and presence of AID protein in one of the RT-PCR-positive cases.

In light of the high somatic mutation load but retained IgM and IgD expression in PCNSL, there appears to be a dissociation between CSR and SHM activity in this lymphoma entity. Interestingly, in extracerebral DLBCL, expression of AID was not associated with an ongoing IgV

A)

B)

Figure 3. Internal deletions in the switch μ region of PCNSL. **A:** PCR amplification of the $S\mu$ region of nine PCNSLs (lanes 2 to 10), Jurkat DNA as a positive control for the full-length product (lane 11), water control (lane 12), and 1-kb DNA ladders (lanes 1 and 13). All PCNSLs exhibited amplification of the full-length product (4.5 kb), whereas in four cases, one additional band (marked with asterisks) and in one case two additional bands were observed. **B:** Graphical representation of switch μ region flanked by the JH6 gene segment, the intronic IgH enhancer, and the $C\mu$ gene. Arrows indicate the primers used for amplification. The shorter products obtained from PCNSL cases 2 and 5 were sequenced. These show deletions of 3.3 kb (case 5; lane 5 in **A**) with a single breakpoint and 1.8 kb (case 2; not shown in **A**) with multiple breakpoints in the switch μ region. Due to the repetitive structure of the switch μ region the short sequence fragments of case 2 cannot be unequivocally assigned to definitive positions. Sequences are available from GenBank under accession numbers AY337410 and AY337411.

hypermutation activity, which is also a characteristic feature of PCNSL,⁸ because subcloning experiments failed to show intraclonal heterogeneity even in DLBCL displaying the highest AID protein amounts.¹⁸ Furthermore, there was no correlation between AID protein levels and the mutation load in extracerebral DLBCL with aberrant SHM of the PIM1, PAX5, RhoH/TTF, and C-MYC genes.¹⁸ Collectively, these data indicate a poor correlation between SHM and AID expression and raise the questions 1) whether low levels of AID may be sufficient for induction and maintenance of SHM but not for CSR; 2) whether SHM and intraclonal diversity have resulted from an earlier phase of clonal expansion, during which the tumor cells have expressed AID, which has been down-regulated at later stages of tumor development; and 3) whether other genetic events may have rendered the tumor cells of PCNSL independent of AID.

What might be the reason for the lack of CSR in PCNSL? First, atypical rearrangements involving downstream CH genes have been described in B-cell lymphomas and may prevent normal CSR. In IgM-expressing follicular lymphoma, which are also characterized by

highly mutated Ig genes as well as ongoing mutation, aberrant CSR events between downstream CH genes with retention of C μ and C δ were frequent.¹⁹ However, other types of B-cell lymphomas lack such recombination events,¹⁹ so that it remains presently unclear whether downstream CH gene deletions may be involved in the rare occurrence or absence of PCNSL with switched isotype.

A second reason could be related to the microenvironment, which may also affect and determine isotype switch of B cells. For example, GC B cells in Peyer's patches of the small intestine preferentially switch to IgA, whereas GC B cells in lymph nodes usually switch to IgG. In this regard, the regional cytokine milieu, in particular interleukin (IL)-4, IL-10, and transforming growth factor- β , may play a critical role in controlling CSR.^{20–23} Under physiological conditions in the CNS, only low levels of cytokines such as IL-10, but no IL-4, can be detected.²⁴ Thus, the tumor cells of PCNSL may originate from (possibly ectopic) GC, which provided support for SHM but not for isotype switch.

Third, the lack of CSR in PCNSL might reflect derivation of the malignant intracerebral B cells from a distinct subset of B cells destined not to undergo CSR in the GC. A considerable fraction of human somatically mutated (memory) B cells expresses IgM ($\pm$ IgD).^{6,25} Retention of IgM expression on a subset of memory B cells may be advantageous to allow a rapid reaction on further antigenic stimulation, because IgM can strongly activate the complement system. Thus, PCNSL may originate from GC B cells destined to become IgM-expressing memory B cells. Interestingly, several other B-cell lymphoma entities with somatically mutated V genes also mostly show IgM ($\pm$ IgD) expression, including Burkitt lymphoma, MALT lymphoma, and extracerebral DLBCL.²⁵

Finally, we speculated that the lack of CSR in the PCNSL could be due internal deletions within the S μ region, which have been identified in some normal and malignant IgM-expressing B cells.^{17,19,26} Because the S μ region is important for CSR, the deletions may impair CSR and hence cause isotype stabilization.¹⁷ Applying a long-distance PCR approach to amplify the S μ region, we detected shortened products in 7 of the 11 cases analyzed. In one of the cases, two distinct shortened amplicates were obtained, suggesting internal S μ deletions on both IgH alleles. Somewhat surprisingly, in the six other cases with internal S μ deletions, we obtained one shortened and one full-length PCR product. We could not clarify whether in these cases the S μ regions with internal deletions are located on the heavy chain allele expressed. It may well be that in some of the cases, the nonproductive allele is involved in chromosomal translocations targeted to the S μ region²⁷ or that a second internal deletion could not be amplified, because it encompassed one of the primer binding sites. Interestingly, genomic deletions within the S μ region have also been detected in IgM-expressing B-CLL and follicular lymphomas, and there is evidence that these deletions preferentially occur on the productively rearranged heavy chain allele.^{19,26} This would argue in favor of the idea that also in PCNSL, the internal S μ deletions are often located on

the IgH allele expressed and, therefore, might explain the absence of CSR in such cases.

In conclusion, the present study demonstrates that PCNSLs only rarely express AID, which may indicate that the intraclonal V gene diversity observed in a fraction of cases mainly occurs in early stages of lymphoma proliferation, when AID may still have been expressed. Moreover, PCNSLs consistently express IgM ($\pm$ IgD) and do not undergo CSR, although the high load of somatic V gene mutations suggests significant AID expression in the tumor precursor cells. The absence of CSR may be due to several reasons, but importantly, we detected internal S μ deletions in 7 of 11 cases as a potential cause for impaired CSR.

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