Activation-induced deaminase in B cell non-Hodgkin’s lymphomas

Expression of activation-induced cytidine deaminase
in human B cell non-Hodgkin’s-lymphomas

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Activation-induced deaminase in B cell non-Hodgkin`s lymphomas

Abstract

Activation-induced cytidine deaminase (AID) induces somatic hypermutations (SHM), class switch recombination (CSR) and immunoglobulin gene conversion in B-lymphocytes. Here we report for the first time the expression of AID in normal human B lymphocytes and in B cell non-Hodgkin`s lymphomas (B-NHL). AID mRNA expression in man is restricted to the CD19^+CD38^+IgD^` germinal center cells, namely the CD19^+CD38^+CD44^` centroblasts. After in vitro stimulation of naive human B cells by CD40-L and IL-4, AID mRNA is strongly induced for only 48 h. In a survey of human B-NHL, AID was found to be constitutively expressed in follicular lymphomas and in diffuse large B-cell lymphomas, but to be absent in B precursor lymphoblastic leukemia, in mantle cell lymphoma and in plasma cell myeloma. In B cell chronic lymphatic leukemia, in immunocytoma and in extranodal marginal zone B cell lymphoma of MALT, AID mRNA was expressed only in some samples. In follicular lymphomas and diffuse large B cell lymphomas, the expression of AID mRNA was coincident with the presence of SHM in the variable region exons of the immunoglobulin heavy chain gene. In human B-NHL, the AID mRNA is spliced into four different variants, but does not contain point mutations. Thus, AID which is highly regulated during normal B cell development is constitutively expressed in human germinal center B-NHLs and also in subsets of non-germinal center B-NHLs. This constitutive expression of AID may promote illegitimate DNA recombinations and somatic mutations in B-NHLs.

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Introduction

Specific chromosomal translocations are a genetic hallmark of lymphoid malignancies in non-Hodgkin’s lymphomas (NHL). The immunoglobulin gene loci are involved in most of these translocations in B cell NHL, suggesting that mistaken maturation of the immunoglobulin genes may contribute to lymphoma formation (1,2). During B cell development the immunoglobulin genes are altered by three distinct mechanisms: In B lymphoblasts, V(D)J joining rearranges the immunoglobulin-gene variable (V), diversity (D) and joining (J) segments to produce the primary antibody repertoire; this well defined process is mediated by the recombination activating genes 1 and 2 (3,4). After antigen encounter, an effective secondary antibody response is achieved by somatic hypermutations (SHM) of the variable region exons and by class switch recombination (CSR) that replaces the μ constant region with one of the downstream located constant regions (5,6). Activation-induced cytidine deaminase (AID) is indispensable for SHM, CSR and immunoglobulin gene conversion in B cells (7-10). AID is a close homologue of APOBEC-1, the catalytic subunit of the apo B mRNA editing-complex; AID and APOBEC-1 are located in tandem on chromosome 12 and apparently have arisen by gene duplication (11-14). APOBEC-1 is an RNA-dependent cytidine deaminase that specifically deaminates C_{6666} in the apo B mRNA together with the APOBEC-1 stimulating protein (12,15-18). In AID deficient mice, both SHM and CSR are completely abolished leading to a severe defect of the humoral immune response (8). In human patients with the autosomal recessive form of the hyper-IgM syndrome (HIGM2) various mutations in the human AID gene have been found that cripple AID function (7). In these patients not only CSR is absent, but also SHM is strongly reduced (7). In fibroblasts, the expression of AID is sufficient to mediate CSR of an artificial switch substrate and SHM in an actively transcribed artificial gene substrate (19,20). Expression of AID in E. coli leads to a
Activation-induced deaminase in B cell non-Hodgkin’s lymphomas

hypermutator phenotype with nucleotide transitions at dC/dG in a context dependent manner (21). Moreover, expression of a bacteriophage derived inhibitor protein of uracil-DNA glycosylase in chicken DT40 cells that lack the repair gene XRCC2 shifts the pattern of SHM from predominantly transversions to transitions (22). These recent results indicate that AID may simply act by deamination of cytidines in the DNA (21,22).

As APOBEC-1 transgenic animals develop hepatocellular carcinomas (23), we reasoned that also AID may be a potential oncogene. Here we demonstrate that AID is constitutively expressed in follicular lymphomas (FL) and diffuse large B cell lymphomas (DLBCL), while in normal B cell development the expression of AID is highly regulated and restricted to germinal center B cells. This constitutive expression of AID may contribute to NHL formation.

Methods

Isolation of B-cell populations from human tonsils:

Human non-malignant tonsils were obtained with written consent from adult patients undergoing tonsillectomy. The tonsil specimens were minced and the single cell suspension was filtered through a 100 µm filter (Filcon, DAKO, Denmark). Lymphocytes were isolated by gradient centrifugation using Ficoll-Paque™ medium (Amersham Pharmacia Biotech, Uppsala, Sweden). Total B cells were isolated with anti-CD19 using immunomagnetic beads (DynalBiotech®, Oslo, Norway) and subsequently fractionated into the following fractions: naive B-cells (CD19⁺CD38⁻IgD⁺), naive germinal center cells (CD19⁺CD38⁺IgD⁺), germinal center cells (CD19⁺CD38⁺IgD⁻) and memory cells (CD19⁺CD38⁻IgD⁻). The CD19⁺CD38⁺ cells were fractionated into CD19⁺CD38⁺CD44⁻ centroblasts and CD19⁺CD38⁺CD44⁺ centrocytes using anti-CD44. The purity of the cell fractions was generally above 95%. Total RNA was isolated from 1X10⁸ cells with Trizol® (Invitrogen). Single-stranded cDNA was synthesized using 500 µg total RNA and a
Activation-induced deaminase in B cell non-Hodgkin’s lymphomas

commercially available random priming synthesis kit (Superscript First Strand Synthesis Kit, Invitrogen).

Isolation of naive human B cells by negative selection and in vitro stimulation with CD40L:

5-10X10^8 naive IgM+ B cells were prepared by negative selection. Briefly, single cell suspensions of B cells prepared from human tonsils were resuspended in HBSS with 5% fetal bovine serum (FBS) and subsequently centrifuged for 6 min at 1600 rpm at 4°C. The cells were resuspended in HBSS/5%FBS (5X10^7 cells/ml), mixed with biotin conjugated antibodies against CD2, CD3, CD14, CD16, CD33, CD56, glycophorin A, IgG and IgA (Stem Cell Technologies, Vancouver, Canada) (100 µl cocktail per ml) and incubated on ice for 45 min. Magnetic colloid coupled to streptavidin (60 µl per ml) was added, and the incubation was continued for another 60 min. Approximately 5X10^8 cells in antibody cocktail plus magnetic colloid were applied to a 0.9 cm preparative metallic MACS column, and the IgM/IgD+ cells were eluted with HBSS/5%FBS. The cells were pelleted for 6 min at 1600 rpm and resuspended in RPMI 1640 plus 10% FBS at a density of approximately 1X10^6 cells/ml. These human naive B cells were stimulated for various times with 2,7 mg/ml of plasma membranes prepared from Sf21 insect cells stably infected with a human CD40L-expressing recombinant baculovirus (CD40L-BV) in the absence or presence of 40 ng/ml IL4 (Pharmingen).

Lymphoma tissue specimens: For all lymphomas investigated, fresh and paraffin-embedded samples were available. Fresh tissue specimens were obtained during surgery, immediately shock frozen in liquid nitrogen and kept at –80°C until use. The diagnoses of the lymphoma subtypes were established by the reference pathologists of the Lymph Node Registry Kiel on the basis of standard histopathology with additional immunohistochemical stainings with mouse monoclonal antibodies using the alkaline phosphatase-antialkaline phosphatase (APAAP) technique. Informed consent was obtained from all patients analyzed in this study. All immunoreagents
Activation-induced deaminase in B cell non-Hodgkin’s lymphomas

were purchased from DAKO (Hamburg, Germany), except for CD20, Ki-B3 and Ki-S5 which had been generated in the Lymphoma Registry Kiel.

B precursor lymphoblastic leukemia/lymphoma (cALL) was diagnosed when blasts expressed TdT, CD79a, CD19 or CD20 and CD10. Diffuse large B cell lymphoma (DLBCL) was diagnosed when centroblasts or immunoblasts expressed B-cell marker like CD20 and showed a high proliferation index determined by Ki-S5 staining. Follicular lymphoma (FL) was diagnosed on the basis of a follicular growth pattern with the expression of CD20, CD10 and bcl-2 in the absence of CD5. B chronic lymphocytic leukemia (B-CLL) and mantle cell lymphoma (MCL) showed a predominance of small or middle sized lymphocytes with co-expression of CD20, CD23 and CD5 in B-CLL or co-expression of CD20, CD5 and cyclin D1 in the absence of CD23 in MCL. Extranodal marginal zone B cell lymphoma of MALT (mucosa associated lymphatic tissue) (MALT-lymphoma) was diagnosed on the basis of the diagnostic lymphoepithelial lesions with expression of CD20 in the absence of Ki-B3 or DBA44. The diagnosis of plasma cell myeloma (MM) was established by the presence of plasma cell infiltrates expressing monotypic light chains. Immunocytoma (lymphoplasmacytic lymphoma) (IC) was diagnosed when lymphoplasmacytoid cells expressed CD20 and IgM with intracytoplasmatic monotypic light chains, and the lymphoma lacked features of other entities e.g. follicular growth or marginal zone features. Based on the Kiel Classification lymphoplasmacytic lymphoma included clonal plasma cells determined by clonal expression of one immunoglobulin light chain. Lymphocytoid immunocytoma contained polyclonal plasma cells. This group of lymphomas is classified as B-CLL in the WHO classification. All samples were completely composed of lymphoma tissue and contained no portions of adjacent tissue. RNA was prepared from 5-7 slices of 7µm using TrizolR (Invitrogen). cDNA pools of the individual lymphoma specimens were synthesized using 1 µg total RNA and a commercially available kit with random primers (SMART TM RACE cDNA Amplification Kit, Clontech).
Activation-induced deaminase in B cell non-Hodgkin’s lymphomas

Oligonucleotides: The following oligonucleotides were used:

AID 1 (sense, nt 48 – 78, GAGGCAAGAAGA CAC TCT GGA CAC CAC CAC TAT)
AID 2 (antisense, nt 408 – 381, GCGGTGAAGATCCTACGGCTAGG TTG)
AID 3 (sense, nt 328 – 358, AGGCCCTGCTACGACTTGCC CGACATGTG)
AID 4 (antisense, nt 681 – 654, AGTTGCTAT CAA AGT CCCAAA GTA CGA)
AID 5 (sense, nt 11 – 40, TAATTGAAG TGAGAT TTTTCT GGCCTGAGA)
AID 6 (antisense, nt 720 – 690, CTTACGCAGAGATAT TTC ATC GTTGCGAC)
AID 7 (antisense, nt 520 – 490, CCAGCAGATAAAA ATA ATC TTTGAA GGTCAT)
AID 13 (antisense, 232 – 203, CTTATT GCGAAGATA ACC AAA GTCCAGTGA)
AID 14 (sense, intron 821 – 8230, GGA GCC GAA ATT AAA GAT TAG AAG CAG AGA)
AID s-1 (sense, nt 200 – 232, TTTTCATGGAG TTTGGTTTAT TTTCCG)
AID as-1 (antisense, nt 520 – 485, CCAGCAGATAAAA ATA ATC TTTGAAAGGT)
AID as-2 (antisense, nt 484 – 461, TATTTCAC CCC GGCGCGGTCAG)
ß-Actin s-2 (sense, nt 391-420, CCCCCTGAACCC CAAGGCCAACCGCGA GAA)
ß-Actin (as-2) (antisense, nt 660-631, TAGCCGCGCTCGGTGAGGATCTTCATGAGG)
ß-Actin as-4 (nt 630-601, TAGTCAGTCAGGTCC CGGCCAGCCAGGTCC)
Blimp-1 s-1 (sense, nt 421-444), GAGTAAAGAATA CAT ACC AAA GGG)
Blimp-1 as-1 (antisense, nt 824-801, CATTTCCTCA TGCTCGTGTGCT)
Blimp 1 as-2 (antisense, nt 730-701, TGCAAAAGTCCCGACA ATA CCA CACAAG)
c-myc s-1(sense, nt 594-617, TCTCAACGCAGCAG CTCGCCCAA)
c-myc as-1 (antisense, 893-870, TTGAGGACCAGTGCTGTAGGA)
c-myc s-2 (sense, nt 618-642, GTCTCAGCTCCGCAAGACTCCAG)
Bcl-6 s-1 (sense, nt 248-271, CAA GAA GTT TCT AGG AAA GGC CGG)
Bcl-6 as-1 (antisense, nt 547-524, GATTGATCA CACTAA GGTTGCAAT)
Bcl-6 as (antisense, nt 520-497, ACTGGT CTTGAA AGATGC TATAGA)
Activation-induced deaminase in B cell non-Hodgkin’s lymphomas

**RT-PCR analysis:** 1 µl of the cDNA synthesis mix was used for PCR amplification. PCR reactions were performed as described (24,25) using the following conditions: AID s-1 and AID as-2: 30 cycles of 94°30'', 58° 30'', 72° 30''. AID 3 and AID 7: 30 cycles of 94° 30'', 55° 30'', 72° 30''. AID 5 and AID 6: 30 cycles of 94° 30'', 55° 30'', 72° 30''. Nested PCR with AID 14 and AID 6 and 30 cycles as above. β-actin s-1 and β-Actin as-1: 25 cycles of 94° 30'', 55° 30'', 72° 30''. Bcl-6 s-1 and Bcl-6 as-1: 30 cycles of 94° 30'', 55° 30'', 72° 30''. blimp-1 s-a and blimp –1 as-1: 30 cycles of 94° 30'', 55° 30'', 72° 30''. c-myc s-1 and c-myc as-1: 30 cycles of 94° 30'', 55° 30'', 72° 30''. The PCR products were hybridized with 32P-labeled internal oligonucleotides (25).

Quantitative PCR was performed with a Light Cycler (Roche®) using standard software for quantification as recommended by the manufacturer. The expression levels were normalized to the expression level of β-actin mRNA. The following conditions were used: AID 3 and AID 7: 45 cycles of 95°0'', 55° 5'', 72° 6''. β-actin s-1 and β-actin as-2: 45 cycles of 95° 0'', 58° 5'', 72° 6''. Bcl-6 s-1 and bcl-6 as-1: 45 cycles of 95° 0'', 58° 5'', 72° 6''. blimp-1 s-a and blimp-1 as-1: 45 cycles of 95° 0'', 58° 5'', 72° 6''. The expression levels were expressed in relation to the expression level of β-actin mRNA that were separately determined in each sample.

**Ribonuclease protection assay:** Radiolabeled antisense AID RNA was synthesized as described spanning 222 bases of the AID mRNA sequence (nt 232-10) (25). Total RNA from human lymphoma tissues (10 µg) was coprecipitated with 2X10^4 cpm of α-32P labeled AID antisense RNA probe. Hybridization and RNA digestion with the Ribonuclease Protection Assay II Kit (Ambion Inc. Austin, TX, USA) and the analysis of the protected RNAs on denaturing polyacrylamide sequencing gels was performed as described (25,26).
Activation-induced deaminase in B cell non-Hodgkin’s lymphomas

Analysis of somatic hypermutation of the variable region of the immunoglobulin heavy chain gene: The recombined V(D)J segments of the IgH transcripts of the lymphoma specimens were amplified from the respective cDNA pools by PCR using a set of degenerate oligonucleotides exactly as described (27).

Determination of immunoglobulin heavy and light chain expression: Immunoglobulin heavy chain and light chain expression was determined by standard immunohistochemistry using antibodies against kappa, lambda, IgM, IgG, IgD, IgE and IgA as described (28).

Results

Expression of AID mRNA in human germinal center B cells: AID mRNA was amplified by RT-PCR from naive B cells (CD19+CD38- IgD+), naive germinal center cells (CD19+CD38+ IgD+), germinal center cells (CD19+CD38+ IgD+) and memory cells (CD19+CD38+ IgD+) (Fig. 1). In germinal center B cells, a strong AID PCR product was generated, while a very faint band was detectable in the memory cell fraction (Fig. 1). No RT-PCR products for AID mRNA were detectable in naive B cells and in naive germinal center B cells (Fig. 1). The CD19+CD38+ germinal center cells were further fractionated with anti-CD44 monoclonal antibodies into centroblasts (CD19+CD38+CD44+) and centrocytes (CD19+CD38+CD44+). AID mRNA was strongly detected in the CD44+ centroblasts, but was hardly detectable in CD44+ centrocytes (Fig. 1).

Naive human IgM+ B cells isolated from human tonsils were cultivated for up to 8 days in the presence of IL-4 and plasma membranes of Sf21 cells that had been infected with a CD40-L expressing baculovirus. AID mRNA was strongly induced between day 2 and 3 (Fig. 2 A). At day 4, the levels of AID mRNA strongly decreased (Fig. 2 A). The mRNA of bcl-6 decreased around day 4 and was hardly detectable from day 5 to day 8 (Fig. 2A). In contrast, the mRNA of
blimp-1 was strongly induced around day 5 (Fig. 2 A). The mRNA expression of AID, bcl-6 and blimp-1 in these cells was quantitated by real-time PCR (Fig. 2 B). The increase of AID mRNA at day 3 was approximately 40-fold in comparison to the AID mRNA levels of naive B lymphocytes at day 1 (Fig. 2 B). At day 0, AID mRNA could not be detected by real time RT-PCR. From day 3 to day 4, the AID mRNA decreased by more than 80% (Fig. 2 B). The mRNA of bcl-6 steeply decreased from day 4 to day 5, while the mRNA of blimp-1 increased approximately 50-fold between day 4 and day 6 (Fig. 2 B). When the naive human IgM+ B cells were cultivated in the presence of CD40-L without IL-4, the AID mRNA of AID was induced much later, at day 4 instead of day, and this induction was far less pronounced (data not shown).

Expression of AID mRNA in human B cell non-Hodgkin´s lymphomas: The mRNA expression of AID as well as of c-myc, blimp-1, bcl-6 and β-actin was studied in cALL, in DLBCL, in FL, in MCL, in IC, in MALT-lymphoma, in B-CLL and in MM. Individual mRNA expression profiles for each entity are depicted in Fig. 3. Within this series, AID mRNA was not detected in any of the cases of cALL (n=3), of MCL (n=4) or of MM (n=3). Strong RT-PCR products of AID mRNA were generated in all cases of FL (n=5) and in all cases of DLBCL (n=13). In MALT-lymphoma (n=8), in IC (n=8) and in B-CLL (n=5), a heterogeneous expression pattern was found for AID mRNA, with strong expression in some and absence of expression in other cases. c-myc mRNA was strongly amplified from DLBCL, while blimp-1 was most abundantly expressed in IC, MM and, to less extent, in B-CLL (Fig. 3). Bcl-6 was strongly expressed in IC (Fig. 3). In normal human lymph nodes (n=4), AID mRNA could only be detected by nested RT-PCR, but not by our conventional RT-PCR, indicating that AID is expressed in normal lymphatic tissues at much lower levels as compared to the germinal center B-NHLs (data not shown).

In all AID expressing lymphomas the full length AID mRNA is the most abundant splice form (Fig. 4). Alternative splicing generates three additional AID mRNA variants. In splice variant 1 (sv1), the intron between exon 3 and exon 4 is not spliced extending the open reading
frame for an additional 45 amino acid residues up to the first stop codon in intron 3 (Fig. 4). In splice variant 2 (sv2), exon 4 is spliced leading to an internal deletion of 39 amino acid residues. In splice variant 3 (sv3), an short neo-exon located in intron 3 is used while exon 3 and exon 4 are excluded (Fig. 4). This spliced variant encodes a truncated peptide consisting of the aminoterminus and carboxyterminus of AID linked by 20 amino acid residues encoded by the neo-exon 3 (Fig. 4). In FL, sv2 was the second minor splice variant, while in MALT-lymphoma, sv1 was the second minor variant (Fig. 4 A). In MALT-lymphoma, both sv1 and sv3 are expressed, while in FL and in DLBCL, only sv3 could be found (Fig. 4). Sequencing did not detect points mutations in AID full length cDNAs from FLs (n=3), DLBCL (n=3) and MALT-lymphomas (n=3).

The expression of AID mRNA in human B cell non-Hodgkin’s lymphoma was directly quantitated by ribonuclease protection analysis (RPA). For these experiments an AID antisense RNA probe complementary to exon 1 and 2 was used. Therefore, full length AID as well as the mRNA variants sv1 and sv2 protect an identical fragment of 222 nucleotides. The expected protected fragment of 222 nucleotides was observed with the highest intensity in MALT, followed by FC and DLBCL (Fig. 5). Quantification of AID mRNA based on the expression of β-actin mRNA revealed that in this particular MALT-lymphoma the AID mRNA levels were approximately tenfold higher than in FL. In the samples of B-CLL and of immunocytoma used in this experiment, no AID mRNA was detectable by RPA (Fig. 5).

**AID mRNA expression, somatic hypermutations and class switch recombination in human B cell non-Hodgkin’s lymphomas:** Next, we studied somatic hypermutation (SHM) of the recombined V(D)J segment and determined the subtype of the constant region of the immunoglobulin heavy chain (IgH) gene in MCL (n=3), in FL (n=3), in DLBCL (n=3) and in B-CLL (n=3) in correlation to the expression of AID mRNA (Fig. 6). In MCL, no AID mRNA is expressed, no somatic hypermutation of the variable region is detectable and the µ constant region of the IgH gene is expressed (Fig. 6). In contrast, in FL and in DLBCL that both express
Activation-induced deaminase in B cell non-Hodgkin’s lymphomas

AID mRNA, the variable region of the IgH gene is hypermutated (Fig. 6). In most cases of FL, the IgH gene locus has not undergone isotype class switch recombination (CSR), and mainly IgM is expressed (Fig. 6). In contrast, in most cases of DLBCL, CSR of the IgH gene has occurred and IgG is expressed (Fig. 6). In the B-CLLs investigated, CSR had not occurred and somatic hypermutation was either absent or very low (Fig. 6).

As not all DLBCL are thought to be derived from germinal center cells, we analyzed a total of 13 cases of DLBCL and stratified them according to their expression of CD10 as detected by immunohistochemistry that can be used to discriminate the germinal center B cell-like type of DLBCL from the activated B cell-like type (29). Five of these DLBCL were positive for CD10, and eight were negative (Table 1). AID mRNA was detected in all five CD10 positive and all eight CD10 negative DLBCLs (Table 1). Three of the CD10 positive DLBCLs had developed from confirmed FLs, while none of the CD10 negative cases had progressed from a FL (Table 1). SHM were detected in 3 out of 3 cases of the CD10 positive DLBCL of which we sequenced the variable region of the immunoglobulin heavy chain gene, and in 5 out of 5 of the CD10 negative DLBCLs (Table 1).

The expression of AID and blimp-1 was also studied in 7 MALT-lymphomas and in 7 immunocytomas (Fig. 7). In the MALT-lymphomas, a rather great variation of AID mRNA expression was observed with relatively high AID expression in three, and low or no expression of AID mRNA in five cases (Fig. 7). The expression of blimp-1 is inversely correlated to the expression of AID in MALT lymphomas, and to less extent, in immunocytomas (Fig. 7). Thus, the lymphomas with high level expression of blimp-1 demonstrated less AID mRNA, and high level expression of AID mRNA was linked to low amounts of blimp-1 (Fig. 7). In the MALT lymphomas, SHM was present in all cases with the exception of two cases in which no IgH transcripts could be detected. Interestingly, the MALT-lymphoma with the high level expression of AID mRNA (lane 2 and Fig. 5) demonstrated only low levels of SHM (3,2% base changes in the variable region), and expressed IgM (Fig. 7). While AID mRNA was absent in two and expressed in five ICs, SHM and CSR could not be detected in the ICs with the exception of one
Activation-induced deaminase in B cell non-Hodgkin’s lymphomas

sample (lane 9) (Fig. 7). Notably, this IC with low level SHM did not express AID, while the ICs with AID expression did not demonstrate SHM (Fig. 7). Therefore, in MALT-lymphoma and in ICs no correlation of AID expression with the presence of SHM in the IgH variable regions is observed. In addition, AID expression was studied in the blood lymphocytes of 10 additional patients with B-CLL. In 7 of these patients, AID mRNA was detected in blood lymphocytes, while in the other 3 AID mRNA was undetectable (data not shown). As in IC, no correlation of AID expression and SHM was found in the blood lymphocytes of these 10 patients with B-CLL.

Discussion

This investigation demonstrates for the first time that AID is highly regulated in normal human germinal center B cells, namely the CD44⁻ centroblasts, but is constitutively expressed in FL and DLBCL. AID mRNA is absent in cALL, in MCL and in MM. In MALT-lymphoma, in IC and also in B-CLL, AID mRNA can be detected only in a subset of cases. Further studies with more patients will be required to investigate whether the expression of AID in these cases is associated with a different growth phenotype, a different response to treatment or even a different clinical outcome.

Recent studies have demonstrated the pivotal role of AID for the induction of both SHM and CSR that mark the transition of the primary humoral immune response to the secondary phase of a mature, highly specific antibody production (10,30,31). The identification of AID enables to study these events at a molecular level. We started to investigate AID expression in normal human germinal center B cells and B cell NHLs as AID might be a pacemaker for lymphoma development and malignant progression. This assumption is supported by the finding of AID induced mutations in E. coli demonstrating that AID alone is sufficient to confer a mutator phenotype (21). Moreover, aberrant hepatic overexpression of the AID homologue
APOBEC-1 induces hepatocellular carcinomas, indicating the oncogenic potential of these genes (23). Previous studies in normal human B cells and in B-NHLs have shown that SHM does not only occur in the variable region of the IgH gene locus, but also in other genes specifically expressed in B cells such as bcl-6 (32-35). In DLBCL, a loss of specificity for SHM and DNA recombination events is observed with respect to the normal B cell that could well be caused by the observed constitutive expression of AID (35).

During normal B cell maturation AID expression is highly restricted to only a short period of time. The constitutive expression of AID in B-NHLs must be due to an autocrine or paracrine secretion of the required growth factors by the neoplastic B cells or by lymphoma infiltrating T cells, or alternatively by a constitutive activation of the AID promoter. Current experiments in our laboratories try to address this important issue. If indeed AID was a pacemaker of B NHL formation, measures to reverse the expression of AID would be an obvious option for treatment or prevention of germinal center B NHLs.

Alternative splicing generates three AID variants in addition to the full length protein. Whether or not these splice variants have a biological role remains to be investigated. The regularly spliced mRNA of AID is the most prevalent AID transcript not only in normal, but also in neoplastic B cells, and AID mRNA remains unmutated in human B-NHLs. Thus, in B NHLs the expression of the AID gene is altered, but the function of the encoded AID protein is unchanged. With respect to the recent results on AID function in E. coli it is possible that the normal AID protein acts as a mutator in neoplastic human B cells (21,35).

The expression of AID in DLBCL and FL was coincident with the occurrence of SHMs in the variable region exons of the IgH locus. On the contrary, the absence of AID expression in MCL was associated with a lack of both CSR and SHM. In DLBCL, the expression of AID is predictable for SHM and CSR and may become a molecular marker for these processes. AID
Activation-induced deaminase in B cell non-Hodgkin`s lymphomas expression was found in all cases of DLBCL studied regardless of whether or not CD10 protein was expressed, or whether the DLBCL developed from a previous FL or not. Currently, we are developing a monoclonal antibody directed against AID that will allow a much easier determination of AID protein expression in human B-NHLs.

In MALT-lymphoma, in IC and in B-CLL a great variation of AID expression was observed, and no correlation of AID expression with SHM and CSR could be demonstrated. A striking negative correlation of AID mRNA to blimp-1 mRNA was observed in MALT-lymphomas. With respect to these two markers, two entities of MALT-lymphomas can be defined that are characterized either by expression of AID and absence of blimp-1 or the *vice versa* situation. This assumption is in accordance with recent results on blimp-1 action in human B cell lines and mouse splenic B cells (36). Blimp-1 extinguishes the gene expression for germinal center B cell function by repressing several transcription factors such as Spi-B and Id3 and induces plasma cell genes such as XBP-1 (36). The results of our investigation indicate that certain subsets within the MALT lymphomas, the ICs and the B-CLLs can be defined solely based on the expression of AID. Further studies with more patients are required to analyze whether the expression of AID in these lymphomas correlates to a different clinical outcome of the afflicted patients. Taken together, the results of our study suggest that AID indeed may be a potent oncogene for B cell NHL development. Besides more extended clinical studies, this hypothesis should now be tested experimentally using transgenic animals.

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Activation-induced deaminase in B cell non-Hodgkin’s lymphomas


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Activation-induced deaminase in B cell non-Hodgkin`s lymphomas


Activation-induced deaminase in B cell non-Hodgkin’s lymphomas


Figure legends
Figure 1: Expression of AID mRNA in human germinal center B lymphocytes. Total human B cells were fractionated by immunoaffinity purification into naive B cells (CD19⁺CD38⁺IgD⁺), naive germinal center cells (CD19⁺CD38⁺IgD⁺), germinal center cells (CD19⁺CD38⁺IgD⁺), memory cells (CD19⁺CD38⁺IgD⁺), centroblasts (CD19⁺CD38⁺CD44⁺) and centrocytes (CD19⁺CD38⁺CD44⁺). cDNA pools were synthesized from total RNA, and the cDNAs of AID (nt 328-520) and β-actin (nt 391-660) were amplified by PCR. The PCR products were separated by agarose gel electrophoresis and hybridized with internal radiolabeled oligonucleotides.

Figure 2: Induction of AID mRNA in human naive B cells by CD40-L and IL-4. Human naive B cells were cultivated in the presence of CD40-L and IL-4 for up to 8 days. The mRNA of AID (nt 200-520), bcl-6 (nt 248-547), blimp-1 (421-824) and β-actin (391-660) was amplified by PCR from cDNA pools, and the PCR products were separated by agarose gel electrophoresis and hybridized with an internal radiolabeled oligonucleotide (A). The mRNA of AID, bcl-6 and blimp-1 was measured by real-time PCR using a Ligh Cycler (Roche®) with the primer pairs as above and normalized to the expression of β-actin mRNA (B).

Figure 3: Expression of AID mRNA in human non-Hodgkin’s lymphomas. cDNA pools were prepared from B precursor lymphoblastic leukemia/lymphoma (cALL), diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), mantle cell lymphoma (MCL), immunocytoma (IC), extranodal marginal zone B-cell lymphoma of MALT (MALT-lymphoma), B cell chronic lymphatic leukemia (B-CLL) and multiple myeloma (MM). The cDNA of AID (nt 200-520), c-myc (nt 594-893), blimp-1 (nt 421-824), bcl-6 (nt 248-547) and β-actin (nt 391-660) was amplified by PCR. The PCR products were separated by agarose gel electrophoresis and hybridized with an internal radiolabeled oligonucleotide. A representative autoradiograph with the PCR products of one individual tissue sample for each NHL entity is shown.
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**Figure 4: Alternative splicing of AID mRNA in human non-Hodgkin`s lymphoma.** The full-length AID mRNA (nt 11-720) was amplified by PCR from cDNA pools of human NHLs, and the PCR products were hybridized with an internal oligonucleotide (A, upper panel). The AID splice variants sv-1 and sv-2 were amplified by half-nested PCR using a specific oligonucleotide (AID14) complementary to the neo-exon in intron 3 (A, lower panel). The amino acid sequence of the four different splice variants of the human AID gene is shown (B).

**Figure 5: Ribonuclease protection for AID mRNA in human non-Hodgkin`s lymphoma.** Total RNA (10 µg) from human NHLs was hybridized with α-32P labeled AID antisense RNA probe (nt 232-10). After RNA digestion the protected AID antisense RNA was analyzed on a denaturing polyacrylamide sequencing gel. As internal standard, ribonuclease protection was performed for β-actin mRNA (lower panel).

**Figure 6: Expression of AID mRNA, somatic hypermutations and class switch recombination of the IgH gene in germinal center non-Hodgkin`s lymphoma.** cDNA pools were synthesized from total RNA of mantle cell lymphoma (MCL) (n=3), follicular lymphoma (FL) (n=3), diffuse large B cell lymphoma (DLBCL) (n=3) and B chronic lymphatic leukemia (n=3). AID (nt 328-520) and β-actin cDNA (nt 391-660) were amplified by PCR and hybridized with a radiolabeled internal oligonucleotides. The recombined V(D)J segments of the IgH transcripts were amplified using a set of degenerate primers and were subsequently sequenced. Point mutations of less then 3% in comparison with the published sequences were regarded as somatic hypermutation negative (-), point mutations of more than 3%, but less than 6% were regarded as somatic hypermutation positive (+), and point mutations of more than 6% were regarded as strong positive for somatic hypermutation (++). Immunoglobulin heavy chain expression was determined by standard immunohistochemistry.
Figure 7: Expression of AID mRNA, somatic hypermutations and class switch recombination of the IgH gene in MALT-lymphoma and immunocytoma. cDNA pools were synthesized from total RNA of MALT-lymphomas (n=7) and immunocytomas (n=7). AID (nt 200-520), blimp-1 (421-824) and β-actin cDNA (nt 391-660) were amplified by PCR and hybridized with a radiolabeled internal oligonucleotide. The recombined V(D)J segments of the IgH transcripts were amplified using a set of degenerate oligonucleotides as primers and subsequently entirely sequenced. Point mutations of less then 3% in comparison to the published sequences are defined as somatic hypermutation negative (-), point mutations of more than 3%, but less than 6% are defined as somatic hypermutation positive (+), and point mutations of more than 6% are defined as strong positive for somatic hypermutation (++). Immunoglobulin heavy chain expression was determined by standard immunohistochemistry.

Table 1: CD10 protein, AID mRNA and somatic hypermutations of the IgH gene in diffuse large B cell lymphomas. In a total of 13 diffuse large B cell lymphomas (DLBCL), CD10 protein expression was analyzed by immunohistochemistry. AID mRNA expression was analyzed by RT-PCR. Three of the CD10+ cases had progressed from a documented FL. The variable region exons of the IgH gene were sequenced in 3 of the CD10+ and in 5 of the CD10- cases.
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Figures

Fig. 1

![Fig. 1 Image]

Fig. 2 A

![Fig. 2 A Image]
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Fig. 2 B

- **AID**
- **bcl-6**
- **blimp-1**
Fig. 3

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Fig. 4

A

B
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Fig. 5

RNA-probe 282 bp
protected fragment 222 bp
(Exon 1 - Exon 2)
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Fig. 6

Fig. 7
Activation-induced deaminase in B cell non-Hodgkin’s lymphomas

Table 1

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Expression of activation-induced cytidine deaminase in human B-cell non-Hodgkin's lymphomas

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