Brief Definitive Report

Somatic Mutation of the CD95 Gene in Human B Cells as a Side-Effect of the Germinal Center Reaction

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Abstract

Somatic hypermutation specifically modifies rearranged immunoglobulin (Ig) genes in germinal center (GC) B cells. However, the *bcl-6* gene can also acquire somatic mutations during the GC reaction, indicating that certain non-Ig genes can be targeted by the somatic hypermutation machinery. The CD95 gene, implicated in negative selection of B lymphocytes in GCs, is specifically expressed by GC B cells and was recently identified as a tumor suppressor gene being frequently mutated in (post) GC B cell lymphomas. In this study, the 5′ region (5′R) and/or the last exon coding for the death domain (DD) of the CD95 gene were investigated in naive, GC, and memory B cells from seven healthy donors. About 15% of GC and memory, but not naive, B cells carried mutations within the 5′R (mutation frequency 2.5×10^{-4} per basepair). Mutations within the DD were very rare but could be efficiently selected by inducing CD95-mediated apoptosis: in 22 apoptosis-resistant cells, 12 DD mutations were found. These results indicate that human B cells can acquire somatic mutations of the CD95 gene during the GC reaction, which potentially confers apoptosis resistance and may counteract negative selection through the CD95 pathway.

Key words: CD95 (Apo-1/Fas) • germinal center • B lymphocytes • somatic hypermutation • apoptosis

Introduction

Somatic hypermutation is a critical mechanism for the generation of diversity in the antibody repertoire (1). This process takes place in antigen-experienced B cells within the germinal center (GC), presumably the so-called centroblasts, and specifically targets rearranged Ig genes. The average mutation frequency of Ig V_H genes in normal human centroblasts is $\sim 6 \times 10^{-2}$ per basepair (2). Despite its high specificity for rearranged Ig genes, the somatic hypermutation machinery was also shown to target the *bcl-6* gene in normal GC B cells (3, 4). Accordingly, (post) GC B cell–derived lymphomas also frequently harbor somatic *bcl-6* mutations (4).

The CD95 (Apo-1/Fas) gene was recently proposed to act as a tumor suppressor gene (5) and is, like *bcl-6*, mutated in a fraction of lymphomas derived from antigen-experienced B cells. In the B lineage, CD95 is specifically expressed at the GC stage of differentiation (6, 7) and may contribute to the propensity of GC B cells to apoptosis. Occupancy of CD95 by CD95 ligand was therefore proposed to represent an important mechanism for negative selection of (e.g., autoreactive) B cells within the GC (7–11). Although autoreactive B cells can bypass negative selection in CD95-deficient *lpr* mice (12), affinity maturation on the basis of positive selection by antigen appeared to remain undisturbed (6). *lpr* mice develop lymphadenopathy and enlargement of liver and spleen and are prone to autoimmunity (12) and B cell lymphoma (13). Germline mutations of the CD95 gene leading to autoimmune lymphoproliferative syndrome (ALPS) and predisposing to B cell lymphoma and other malignancies have been observed in humans as well (5, 14). Notably, the majority of these patients develop follicular hyperplasia and progressive transformation of GCs, leading to profound alterations of the normal GC architecture (15).

Somatic mutations impairing the transduction of the apoptosis signal were observed in a number of lymphoid malignancies (16, 17). Deleterious mutations of exon IX of the CD95 gene coding for the death domain (DD) act in a dominant-negative way, which is likely due to the trimerization of CD95 on the cell surface (14). The DD is a

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highly conserved region that is required and sufficient for the transduction of the death signal (14).

During tumor progression, malignant cells frequently lose their susceptibility to CD95-mediated apoptosis and thus escape immunosurveillance and rejection (e.g., by CD95 ligand expressing cytotoxic T cells; for review see reference 18). In consequence, loss of CD95 function due to somatic mutations should favor immune evasion of malignant cells.

In lymphomas derived from antigen-experienced B cells, mutations of the CD95 gene may reflect increased mutability due to the malignant transformation and defective DNA repair pathways in the neoplastic cells. On the other hand, such mutations may have been acquired by the precursor cell of the tumor clone during the GC reaction. To test the latter possibility, naive, GC, and memory B cells of healthy donors were purified and analyzed for mutations of the CD95 gene.

Materials and Methods

Cell Separation and Flow Cytometry. For single-cell analysis, B cell subsets were purified from reactive tonsils of seven donors (aged from 3 to 41 y). $CD38^+CD77^+$ GC B cells were isolated as previously described (19). Repeating the MACS™ enrichment of $CD77^+$ cells once, a purity of >90% $CD38^+CD77^+$ cells was obtained. From six donors, single $CD38^+CD77^+$ cells were sorted directly into PCR tubes containing 20 μl of 1× Expand High Fidelity PCR buffer (Boehringer Mannheim) on a FACS® 440 (Becton Dickinson). Tonsillar naive and memory B cells were isolated from the flowthrough fraction ($CD77^-$ cells). The fraction of naive B cells was twice depleted from CD27-expressing cells, whereas the memory B cell fraction was twice enriched for CD27 expression by MACS™. Naive B cells ($IgD^+IgM^+CD20^+CD27^-$) and memory B cells ($IgD^-IgM^-Ig\kappa^+CD27^+$) were then directly sorted into PCR tubes.

Cloning Procedure. For an initial experiment, genomic DNA was extracted from naive (IgD^+CD27^-) and GC ($CD38^+CD77^+$) B cells purified from tonsillar tissue of another donor (20) and used for amplification of exon IX of the CD95 gene using Pfu Turbo polymerase (Stratagene) in 35 PCR cycles and cloned into the pGEM-T cloning vector.

Single-Cell PCR. For all sorted cells, a whole genome preamplification step (21) was performed. Aliquots of 4 μl from these reactions were then subjected to two rounds of seminested PCR amplification as previously described. In brief, rearranged V_H genes were amplified using family-specific framework region I V gene primers and two sets of J_H primers in a seminested approach (19).

As depicted in Fig. 1, two regions of the CD95 gene were analyzed by single-cell PCR. Exon IX coding for the DD was amplified using 5′-CAC TAA TGG GAA TTT CAT TTA GA-3′ as external forward, 5′-TGG GAA TTT CAT TTA GAA AAA CA-3′ as internal forward, 5′-TAA TTG CAT ATA CTC AGA ACT GA-3′ as external reverse, and 5′-TAC TCA GAA CTG AAT TTG TTG T-3′ as internal reverse primers in a nested PCR (two amplification rounds of 35 and 45 cycles at an annealing temperature of 55°C). A 750-bp fragment encompassing the 5′ region of the CD95 gene (Fig. 1) was amplified using 5′-ACC ACC GGG GCT TTT CGT GA-3′ as external forward, 5′-TGA GCT CGT CTC TGA TCT CG-3′ as internal forward, 5′-TAT CTG TTC TGA AGG CTG CAG-3′ as external reverse, and 5′-CGG AGC GGA CCT TTG GCT-3′ as internal reverse primers in a nested PCR at an annealing temperature of 58°C. Sequence analysis for cases II and III revealed that the somatic mutations within the 5′ region of the CD95 gene clustered to a genomic region flanking the first exon. Thus, further analysis focused on a 510-bp region using 5′-CTT CGC ATC AAG GCC CAA GAA-3′ and 5′-AAG GCC CAA GAA AAG CAA GTC-3′ as external and internal reverse primers, respectively. PCR products were gel purified and directly sequenced using the BigDye Terminator cycle sequencing kit and an automated sequencer (ABI 377; Applied Biosystems). To rule out Taq DNA polymerase errors during the first and second rounds of PCR, 10 CD95 mutations were reproduced by amplification of the respective PCR products from two independent aliquots of the whole genome preamplification reactions.

Selection of GC B Cells Carrying a Mutated CD95 DD. After MACS™ purification, the $CD38^+CD77^+$ GC B cells from donors II–V were suspended in undiluted supernatants conditioned by the lymphoblastoid marmoset cell line B95.8 that releases EBV into the culture fluid. The GC B cells were cultured overnight at 37°C (humidified 5% CO_2 atmosphere) in the presence of EBV (22) and then transferred into multiple wells of a microtiter plate at a density of 10^5 cells per well. In accordance with previous findings (22), we showed in an independent experiment that the number of viable cells remained unchanged in these cultures, whereas virtually all GC B cells died in the absence of EBV. Thus, in the presence of EBV, the surviving B cells were rescued from apoptosis presumably by EBV-dependent antiapoptotic signals. The EBV-containing culture medium was supplemented with an agonistic antibody to CD95 (Beckman Coulter) at a concentration of 100 ng/ml. After 16 h of incubation, >90% of the GC B cells became apoptotic in the presence yet not in the absence of the agonistic anti-CD95 antibody, as assessed by membrane staining for annexin V (CLONTECH Laboratories, Inc.; see Fig. 2). In line with earlier findings (23), EBV confers prolonged survival to in vitro–cultured B cells but does not affect sensitivity to CD95-mediated apoptosis. Single, nonapoptotic GC B cells (annexin V–negative, propidium iodide–negative, $CD38^+$) were directly sorted into PCR tubes.

Statistics. The levels of statistical significance were calculated using the χ^2 test for rows and columns of contingency tables. $P < 0.05$ was considered statistically significant.

Results

To investigate whether normal B cells can acquire somatic mutations of the CD95 gene during the GC reaction, in an initial experiment, exon IX of the CD95 gene was amplified, cloned, and sequenced from genomic DNA of naive and GC B cells ($CD38^+CD77^+$ centroblasts). In 21 clones from naive B cells, 1 mutation was found, whereas in 30 clones from GC B cells, 4 clones were mutated (Table I). This prompted us to investigate CD95 mutations in single naive ($IgD^+IgM^+CD27^-$), GC ($CD38^+CD77^+$ centroblasts), and memory ($IgD^-IgM^-Ig\kappa^+CD27^+$) B cells from six further donors. The single-cell approach was chosen because direct sequencing of PCR products from single cells circumvents DNA polymerase–introduced mutations, which might pose a problem in a low-level mutation analysis when PCR products are

cloned before sequencing. For each B cell subset, single cells were isolated by fluorescence-activated cell sorting from reactive tonsils of six donors (aged 3–41 y). From these cells, a 750-bp fragment of the CD95 gene encompassing 5′ untranslated and coding regions of exon I and a p53 responsive enhancer within the first intron was analyzed. This region (here collectively termed 5′ region [5′R]; Fig. 1) is located ~100 bp downstream of the transcription initiation site and was selected in analogy to the *bcl-6* gene that was previously shown to harbor a major cluster of somatic mutations within its 5′ regulatory regions (4). In addition, a 440-bp fragment comprising exon IX coding for the DD of CD95 was analyzed (Fig. 1). The purity of the sorted B cell subsets was verified by amplification and sequencing of Ig V_H gene rearrangements from 8 naive B cells (none mutated), 21 GC B cells (all mutated), and 16 memory B cells (all mutated; Table I).

Analyzing 5′R sequences from 135 B cells from 4 donors, we detected two novel germline polymorphisms at nucleotide positions +275 and +337 in two donors. For the calculation of the mutation frequency, we took advantage of these novel polymorphisms, as they allowed us to

Table I. *Somatic Mutations of the CD95 Gene in Naive, GC, and Memory B Cells*

B cell subset; Phenotype	Donor	Method	Mutated cells*			
			Overall	5′R	Exon IX	Ig V_H
Naive;	I	Cloning	1/21	ND	1/21	0/17‡
$IgD^+IgM^+CD27^-$	II	Single cell	0/19	0/19	0/14	0/4
	III	Single cell	0/12	0/9	0/9	ND
	IV	Single cell	1/11	1/8	0/7	0/1
	V	Single cell	0/14	0/12	0/8	0/3
	VI	Single cell	0/14	ND	0/14	ND
	VII	Single cell	0/14	ND	0/14	ND
	ΣII–VII	**Single cell**	**1/84¶**	**1/48¶**	**0/66¶**	**0/8**
GC;	I	Cloning	4/30	ND	4/30§	13/13‡
$CD38^+CD77^+$	II	Single cell	4/25	3/25	1/9§	5/5
	III	Single cell	3/15	3/14	0/5	4/4
	IV	Single cell	2/15	1/10	1/6§	4/4
	V	Single cell	3/14	3/11	0/2	4/4
	VI	Single cell	0/12	ND	0/12	ND
	VII	Single cell	0/11	ND	0/11	ND
	ΣII–VII	**Single cell**	**12/92‖¶**	**10/60‖**	**2/45¶**	**17/17**
Anti-CD95–resistant GC;	II	Single cell	5/5	3/4	3/5	ND
Annexin V–negative,	III	Single cell	1/7	0/2	1/7	2/2
$CD38^+$	IV	Single cell	2/5	0/4	2/5	ND
	V	Single cell	1/5	ND	1/5	2/2
	ΣII–V	**Single cell**	**9/22‖**	**3/10‖**	**7/22‖**	**4/4**
Memory;	II	Single cell	1/13	1/7	0/9	4/4
$IgD^-CD20^+CD27^+$	III	Single cell	2/8	2/4	0/8	5/5
	IV	Single cell	0/8	0/3	0/8	4/4
	V	Single cell	2/8	2/3	0/8	3/3
	ΣII–V	**Single cell**	**5/37‖**	**5/17‖**	**0/33¶**	**16/16**

*Clones for donor I.
‡Genomic DNAs used in the cloning experiment were already studied in Jungnickel et al. (20).
§All cells/clones harbor replacement or truncating mutations.
‖$P < 0.05$ as compared to naive B cells.
¶$P < 0.05$ as compared to anti-CD95–resistant GC B cells.

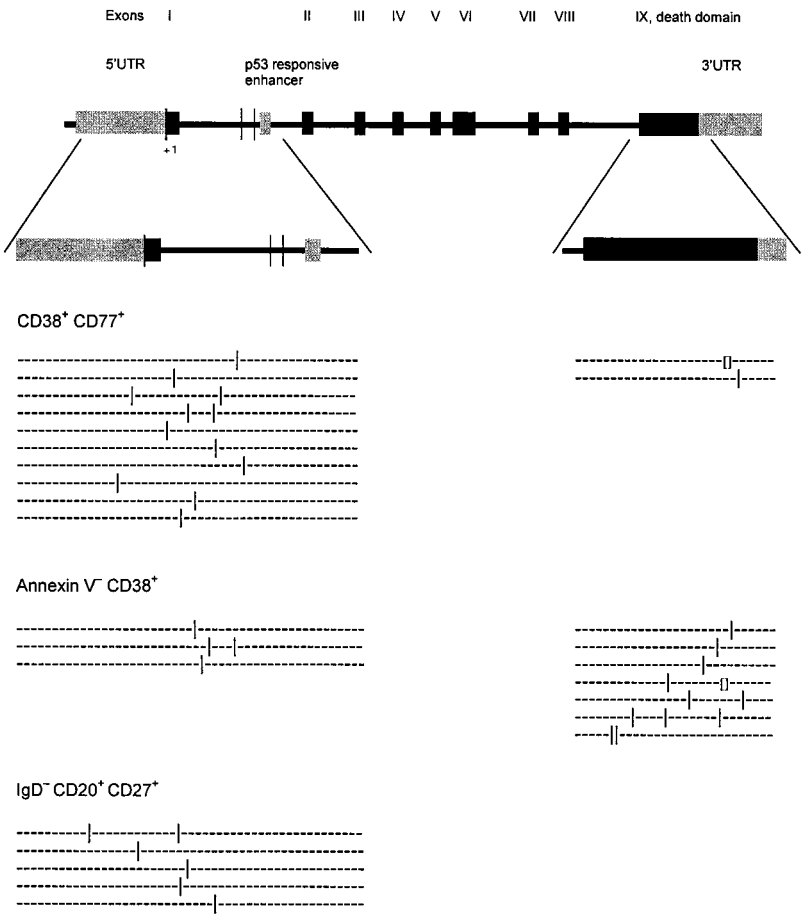

Figure 1. Organization of the CD95 gene and distribution of somatic mutations. The organization of the CD95 gene (comprising exons I–IX) is depicted. From single naive ($IgD^+IgM^+CD27^-$), GC ($CD38^+CD77^+$), anti-CD95–resistant GC (annexin V–negative, $CD38^+$), and memory ($IgD^-CD20^+CD27^+$) B cells of four donors, a 750-bp fragment encompassing 5′ untranslated (gray boxes) and coding (black boxes) regions of the first exon and a p53-responsive intronic enhancer was amplified. From the same cells, a 440-bp fragment containing exon IX coding for the DD of CD95 was amplified. Two novel germline polymorphisms are indicated by vertical lines. Below, the distribution of somatic mutations within the two regions of GC and memory B cells is depicted (point mutations as vertical lines and deletions as brackets).

distinguish between amplification of only one (40% of all cells) or both alleles (60% of all cells) from a single cell (i.e., on average 1.6 alleles were amplified).

Notably, somatic mutations of the 5′R of the CD95 gene were detected in ~15% of GC and memory B cells (Table I). Assuming that also in the noninformative cases on average 1.6 alleles of the CD95 gene were amplified from each cell, an overall mutation frequency of the 5′R in GC and memory B cells of 2.6×10^{-4} per basepair can be calculated. In contrast, in a total of 48 naive B cells, only one mutation within the 5′R was detected (Table I). As none of the 22 mutations within the 5′R (Table II) targeted the first exon or the p53-responsive enhancer (Fig. 1), their functional relevance remains unclear. Mutations within the DD were very rare and only found in GC B cells (mutation frequency in GC B cells 7×10^{-5} per basepair).

To confirm that GC B cells harboring a mutated DD indeed occur within the GC (although at a low frequency)

Table II. *Frequency of CD95 Mutations in Human B Cell Subsets; Single-Cell Analysis from Donors II–VII*

B cell subset; phenotype	Mutations per cell		Mutation frequency	
	5′ region	Exon IX	5′ region	Exon IX
			$\times 10^{-4}$/bp*	
Naive; $IgD^+IgM^+CD27^-$	1/48	0/66‡	0.3	<0.2‡
GC; $CD38^+CD77^+$	12/60	2/45	2.5	0.7
anti-CD95–resistant GC; annexin V–negative, $CD38^+$	4/10	12/22	5.0	7.7
Memory; $IgD^-CD20^+CD27^+$	6/17	0/33‡	3.1	<0.4‡

*Calculation of mutation frequencies is based on average amplification of 1.6 alleles from one cell. Mono- and biallelic amplification was distinguished by germline polymorphisms for the 5′R and by mutations for the DD, respectively.
‡For calculation of mutation frequencies, 0 mutations was set to <1 mutations in 33 and 66 cells.

and to determine whether mutations of the DD have impact on CD95-dependent apoptosis, an in vitro selection experiment was performed (Fig. 2). As GC B cells undergo apoptosis within a few hours under standard cell culture conditions, they were rescued by short-term EBV infection that does not yet lead to lymphoblastoid transformation (22). Confirming previously published data (22), >80% of the GC B cells were still viable after overnight culture. As in vitro–cultured B cells retain their full sensitivity to CD95-mediated apoptosis even after EBV immortalization (23), an agonistic (apoptosis-inducing) antibody to CD95 was added to the GC B cells. After 16 h, >90% of the GC B cells became apoptotic (annexin V–positive) in the presence yet not in the absence of the antibody (Fig. 2). Single annexin V–negative, $CD38^+$ GC B cells were sorted and analyzed for somatic mutations within the 5′R and the DD. In 22 cells, 12 DD mutations were found (7 mutated cells, mutation frequency 7.7×10^{-4} per basepair; Table II). Compared with nonselected GC B cells, the frequency of GC B cells harboring a mutant DD was significantly increased within the anti-CD95–resistant population ($P < 0.01$). Unexpectedly, the frequency of 5′R mutations was slightly increased compared with unselected GC B cells (Table II). Among the 14 DD mutations detected in GC B cells, two mutations creating a preterminal translation stop, two deletions resulting in frameshift, and nine replacement mutations were found. One silent mutation was detected in a GC B cell that also harbored a replacement mutation. Thus, it is indeed possible to selectively enrich GC B cells carrying a mutated DD in vitro, indicating that the DD mutations in GC B cells confer resistance to (or at least delay of) CD95-mediated apoptosis.

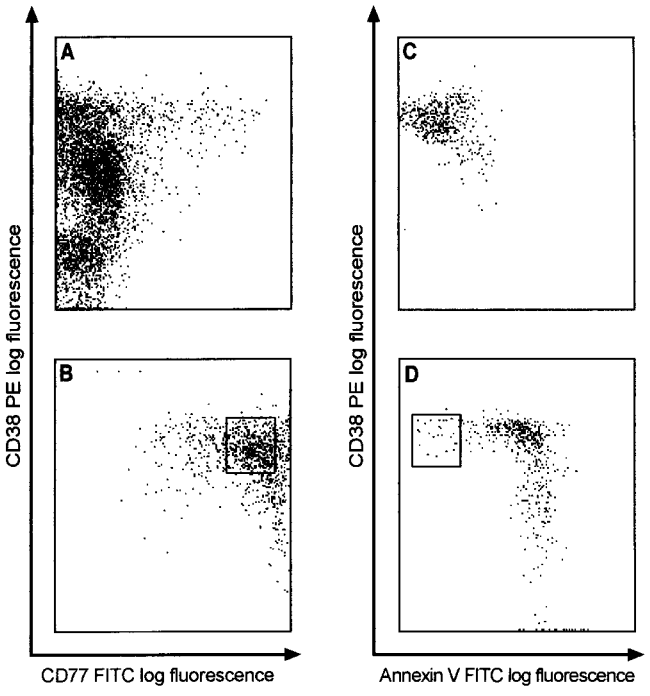

Figure 2. In vitro selection of anti-CD95–resistant GC B cells. Tonsillar mononuclear cells were stained for the GC markers CD38 and CD77 with <5% double-positive cells (A). After MACS™ enrichment for $CD77^+$ cells, the purity of $CD38^+CD77^+$ B cells increases up to >90%. From the gated population, single cells were sorted into PCR tubes for further analysis (B). The purified $CD38^+CD77^+$ GC B cells were incubated with EBV-containing supernatants and cultured overnight. Thereafter, the GC B cells were incubated for 16 h in the absence (C) or presence (D) of an agonistic anti-CD95 antibody at 100 ng/ml. In >90% of the GC B cells, the agonistic anti-CD95 antibody induced apoptosis (positive staining for annexin V; D). From the annexin V–negative, $CD38^+$ GC B cells (D, gate) that had not undergone apoptosis, single cells were sorted into PCR tubes.

Discussion

This study shows that normal human B cells can acquire somatic mutations of the CD95 gene during the GC reaction. CD95 is, like the *bcl-6* gene, specifically expressed at the GC stage of B cell differentiation and frequently mutated in (post) GC B cell lymphomas. In comparison to *bcl-6*, however, the frequency of somatic mutations within the CD95 gene in (post) GC B cells is considerably lower (3, 4). For both genes, it is conceivable that the mutations are introduced as byproducts of somatic hypermutation acting outside the Ig loci. It will be interesting to determine whether this phenomenon is specific for a distinct subset of GC-related genes or can affect any gene expressed concomitantly with the process of somatic hypermutation.

Somatic mutations in rearranged Ig genes and in the *bcl-6* gene are largely confined to a genomic region of ~1.5 kb downstream of the transcription initiation site. This is compatible with the clustering of CD95 mutations within the 5′R (as opposed to mutations within the DD, i.e., >15 kb downstream of the transcription initiation site). As described for both Ig and *bcl-6* genes, a bias for transitions over transversions (26:8) was observed for mutations in the CD95 gene. While somatic mutations are preferentially introduced at RGYW motifs in Ig and *bcl-6* genes (4), this was not evident for the 5′ region of the CD95 gene. However, as not all RGYW motifs in Ig genes are mutational hot spots, this does not argue against an involvement of the hypermutation machinery (for review see reference 2).

From the finding that somatic mutations in the CD95 gene also occur in T cell–derived malignancies and solid tumors (for review see reference 5), it is obvious that other mechanisms can contribute to CD95 mutations. In line with this view, the pattern of CD95 mutations in T cell malignancies (i.e., large deletions rather than point mutations) clearly differs from that described here. As neither T cell neoplasms nor solid tumors have been analyzed for mutations within the 5′R of the CD95 gene so far, we have amplified and sequenced this region from 48 mostly high grade breast cancer samples and found no mutation (Müschen, M., and D. Re, unpublished observation).

A fraction of somatic CD95 mutations, in particular those within the DD, may indeed have significant impact on the fate of a B cell within the GC. Truncating mutations and also many replacement mutations within the DD have been shown to act in a dominant negative manner and are related to a clinical phenotype (ALPS; for review

see reference 5). Using an in vitro selection procedure, this study provides direct evidence for the link between mutations within the DD and resistance to CD95-mediated apoptosis: triggering CD95-mediated apoptosis in vitro resulted in a 10-fold higher frequency of DD mutations in the surviving cells ($P < 0.01$). That B cells harboring an inactivated CD95 gene are indeed also positively selected within the GC is supported by the finding that all mutated DD sequences from GC B cells carried either replacement or truncating mutations (Table I). The observation that mutations within the 5′R were apparently coselected with DD mutations (Table II) might indicate that the mutations within both regions were generated through the same mechanism, namely somatic hypermutation as it is usually targeted to rearranged Ig genes.

Several lines of evidence suggest that CD95 is important for affinity maturation of B cells during the GC reaction: survival signals through B cell receptor engagement and costimulatory CD40 signaling confer resistance to CD95-mediated apoptosis (9, 10, 24) and represent a means of positive selection by antigen (1, 11). Hence, CD95 deficiency in GC B cells due to somatic mutation might allow GC B cells expressing mutant antibodies to bypass the stringent selection mechanisms involved in affinity maturation. While an earlier study of affinity maturation of the antibody response to 4-hydroxy-3-nitrophenyl (NP) in *lpr* mice did not support this possibility (6), recent data of Takemori and colleagues, obtained in the same experimental system, indicate that CD95 indeed plays a role in GC B cell selection (Takemori, T., personal communication).

The process of somatic hypermutation is a potential source of self-reactive antibody specificities (1). Therefore, autoreactive B cells are vigorously counterselected within the GC (25). In CD95-deficient *lpr* mice (12) and humans suffering from ALPS (14), autoreactive B cells producing high levels of autoantibodies accumulate, indicating that negative selection against autoreactivity is impaired (12). In this study, GC B cells that had lost their sensitivity to CD95-mediated apoptosis were detected in five donors at young age (3 to 6 y). Although such mutations were only found at a very low frequency, we can envision that these cells may accumulate during the aging process and represent a potential source for autoantibody production.

Somatic mutations of the CD95 gene were previously found in a number of (post) GC B cell lymphomas including follicular lymphoma, diffuse large B cell lymphoma, anaplastic large cell lymphoma (17), plasmocytoma (16), and Hodgkin's disease (26). The overall frequency of (post) GC B cell lymphomas harboring one or more CD95 mutations is ~16% (for review see reference 5). Given that germline mutations of the CD95 gene are related to lymphoproliferation and B cell lymphoma in humans and mice (13), it was proposed that CD95 acts as a tumor suppressor gene (5). As this study describes that normal GC B cells can also acquire somatic mutations of the CD95 gene, it is unlikely that these mutations already commit a GC B cell to malignant transformation. Under the assumption of a multistep lymphomagenesis, however, deleterious mutations within the DD can favor the persistence of mutant GC B cells until further transforming events take place.

A number of features of GC B cell physiology (e.g., high proliferation rate, somatic hypermutation, class switch recombination) bear an inherent risk of malignant transformation (27). The finding of deleterious CD95 mutations in GC B cells supports the concept that the GC reaction, besides its physiological role, is a potential source of oncogenic alterations predisposing to B cell lymphoma.

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