Loss of Fas (CD95/APO-1) Regulatory Function Is an Important Step in Early MALT-Type Lymphoma Development

Harald Seeberger, Petr Starostik, Stephan Schwarz, Constanze Knörr, Jörg Kalla, German Ott, Hans Konrad Müller-Hermelink, and Axel Greiner

Institute of Pathology, University of Würzburg, Würzburg, Germany

SUMMARY: Fas (CD95, APO-1) mutations were found in autoimmune diseases and some lymphomas, suggesting impairment of Fas-mediated cell death signaling that may cause tumor development. Because mucosa-associated lymphoid tissue (MALT)-type lymphoma B cells recognize autoantigens and proliferate in response to antigen and T cell-mediated signals, it is suggestive that autoreactive B cell lymphoma precursor cells may have escaped the Fas-mediated checkpoint that normally operates in healthy individuals. Using different biochemical, molecular, and functional approaches, we analyzed the Fas signaling in malignant B cells from seven MALT-type lymphomas that were additionally characterized for the t(11;18)(q21;q21) and four gastric diffuse large B cell lymphomas (DLBL). All DLBLs and three of seven MALT-type lymphomas were resistant to Fas-mediated apoptosis in vitro. Moreover, four of five MALT-type lymphomas analyzed and one of three DLBLs analyzed showed mutations in Fas mRNA transcripts but no loss of heterozygosity in the Fas promotor region. Alternative mechanisms of resistance to apoptosis, such as decreased expression of Fas or production of soluble Fas were not operative. Therefore, it is suggestive that a subgroup of MALT-type lymphoma B cells, irrespective of t(11;18)(q21;q21), escape the censoring Fas pathway by mutating and inactivating Fas. This identifies a key regulatory step in early MALT-type lymphomagenesis. (*Lab Invest 2001, 81:977–986*).

Marginal zone B cell lymphomas of mucosa-associated lymphoid tissue (MALT)-type are neoplastic counterparts of normal memory B cells (Harris et al, 1994). The development from naive to memory B cells is closely accompanied and regulated by T cells through Fas/FasL interaction (Nagata, 1997; Suda et al, 1993) taking place in the germinal centers of follicles and leading either to deletion of autoantigen-recognizing B cells or to further differentiation into memory B cells resident in the marginal zone of the follicle (Goodnow, 1996; Rathmell et al, 1996) and plasma cells. Therefore, disruption of the Fas/FasL apoptotic pathway is associated with lymphoproliferative and autoimmune disorders as demonstrated in mice carrying lymphoproliferation (lpr) or generalized lymphoproliferative disease (gld) mutations affecting the genes for Fas and FasL, respectively (Takahashi et al, 1994; Watanabe-Fukunaga et al, 1992), or in humans carrying inherited mutations in the Fas gene leading to autoimmune lymphoproliferative syndrome (Bettinardi et al, 1997; Fisher et al, 1995; Rieux Laucat et al, 1995; Sneller et al, 1997). Because autoantigen receptors and marginal zone B cell expansions are characteristic features of MALT-type lymphoma (Greiner et al, 1998), we searched for a link between tumor development and functionality of the Fas/FasL apoptotic pathway. In addition, the translocation t(11;18)(q21;q21) is the most characteristic chromosomal aberration in extranodal MALT-type lymphoma and may interfere with the regulation of apoptosis. In recent studies, the gene for the human inhibitor of apoptosis protein-1 (HIAP-1) on chromosome 11 was identified as being directly affected by this translocation (Dierlamm et al, 1999). The protein HIAP-1 has been implicated in Fas-induced apoptosis and other death pathways by direct inhibition of some caspases (Deveraux and Reed, 1999; Deveraux et al, 1998; Roy et al, 1997).

Therefore, the malfunction of apoptosis could explain the insensitivity of malignant B cells toward T cell control that may exist in vitro and in vivo (Greiner et al, 1997; Hussell et al, 1996; Knörr et al, 1999). In our study, we measured T cell-induced, Fas-mediated apoptosis in malignant B cells in vitro and compared it with normal memory B cells under the same experimental conditions. We found that resistance to Fas-mediated apoptosis characterizes certain MALT-type lymphomas, and we further examined the mechanisms underlying this phenomenon.

Results

Malignant B Cells of MALT-Type Lymphoma, but Not DLBL, Show Reduced Fas Expression Compared with Normal Memory B Cells

Considering a potential Fas/FasL interaction between B and T cells, we first determined basal Fas

expression by FACS analysis of freshly isolated malignant B cells from the investigated cases of MALT-type lymphoma and compared it with that of normal memory B cells. Basal Fas expression of normal memory B cells was found to be moderate (mean fluorescence intensity [MFI], 22; Fig. 1A). However, malignant B cells of MALT-type lymphoma differed: four cases had very low Fas expression (mean MFI, 4) and three cases showed low Fas expression (mean MFI, 10). In contrast, malignant B cells from diffuse large B cell lymphoma (DLBL) exhibited a very heterogenous Fas expression, ranging from MFI 15 to MFI 55 (mean MFI, 33).

Next, we cocultured B cells with activated T cells for 24 hours and again determined Fas expression of B cells. Normal memory B cells increased Fas expression more than twofold (MFI, 52) under these conditions. A similar inducibility of Fas expression was also found in malignant B cells from MALT-type lymphoma. But here the absolute values of expression were markedly lower (mean MFI, 17 and 23, respectively). In contrast, Fas expression of malignant B cells from DLBL was only slightly enhanced by activated T cells.

Activated T Cells Induce Apoptosis via Fas/FasL Interaction in Normal Memory B Cells, but Not in Most Tumor B Cells

We tried to elucidate the influence of activated T cells on induction of Fas-mediated apoptosis in normal and malignant B cells. Normal memory B cells underwent apoptosis when cocultured with activated T cells (Fig. 1C). Their survival rate decreased drastically from 40% to 14%, resulting in a survival index of 0.35. Malignant B cells from four cases of MALT-type lymphoma showed a comparable decrease in the survival rate. These were exactly the cases with the lowest Fas expression. In contrast, malignant B cells from the remaining three cases of MALT-type lymphoma and from all gastric DLBLs showed constant or even enhanced survival in T cell coculture; they were resistant to T cell-induced apoptosis, as shown in Case 5 (Fig. 1B).

In a control experiment, we added a blocking antibody of Fas receptor (ZB 4; Immunotech, Hamburg, Germany) to a T/B cell coculture. In this assay we found a 50% reduction in the number of dead B cells (Fig. 1D), demonstrating that T cell-induced apoptosis is indeed mediated via the Fas molecule.
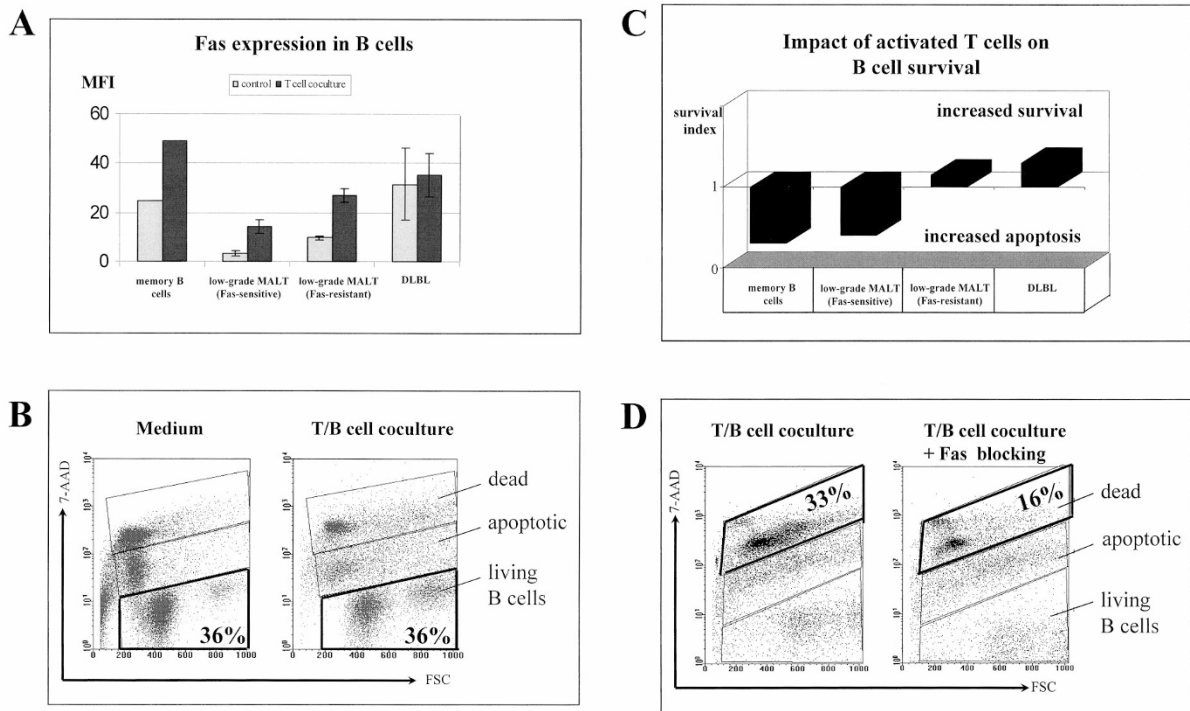

Figure 1.

Influence of activated T cells on Fas expression and survival of malignant B cells from mucosa-associated lymphoid tissue (MALT) lymphoma. A, Activated T cells enhance Fas expression markedly in normal memory B cells and malignant B cells from MALT-type lymphoma (four cases were Fas-sensitive, three cases Fas-resistant), but only slightly in malignant B cells from four cases of diffuse large B cell lymphoma (DLBL) as determined by FACS analysis. MFI, mean fluorescence intensity. Error bars indicate standard deviation of MFI. B, The number of living B cells from a Fas-resistant MALT-type lymphoma (Case 5) is not reduced by T cell influence as measured by FACS analysis of B cells stained with 7-amino actinomycin D (7-AAD). FSC, forward scatter. C, The summary of our findings shows that activated T cells induce apoptosis only in normal memory B cells and in malignant B cells from a subgroup of MALT-type lymphomas (four out of seven cases), indicated as Fas-sensitive. Survival index: percentage of surviving B cells in T cell coculture/percentage of surviving B cells without coculture. D, The control experiment demonstrates that apoptosis is Fas-mediated because Fas-sensitive malignant B cells (Case 3) can be rescued from apoptosis in the presence of an antibody (ZB 4) that blocks the Fas molecule, as measured in FACS analysis of B cells stained with 7-AAD.

Caspases-3 and -8 Are Present in Malignant B Cells Resistant to Apoptosis

We examined poly-ADP ribose polymerase (PARP)-cleavage, a hallmark of apoptosis, by Western blotting and found in normal B cells two bands corresponding to intact and cleaved PARP, both without and with T cell coculture. In Fas-resistant malignant B cells, PARP-cleavage, but not processing of caspases-3 and -8, was abrogated in T cell coculture (Fig. 2A).

We further looked at additional genes involved in pathways of apoptosis or survival. Using reverse transcription-polymerase chain reaction (RT-PCR), we monitored the expression of genes whose products inhibit apoptosis, such as bcl-2 and bcl-xL, or promote apoptosis, such as bcl-xS, bax, p53, and c-myc.

In normal memory B cells, basal expression of bcl-xL, bcl-2, bax, c-myc, and p53 was enhanced by coculture with activated T cells. In contrast, Fas-resistant malignant B cells showed no detectable expression of bcl-xL, but they still showed induction of c-myc and p53 (Fig. 2B). Among the Fas-sensitive malignant B cells, two cases out of four showed no detectable c-myc and p53 expression. In conclusion, there was no specific expres-sion pattern that would characterize either Fas-resistant or Fas-sensitive B cells.

Malignant B Cells Produce mRNA for Soluble Fas (sFas), but No Protein

RT-PCR of the complete coding sequence of Fas yielded not only the expected product of 1008 bp in size, but also a smaller one with 945 bp, which was found in two cases of Fas-resistant MALT-type lymphoma and in two cases of DLBL (Fig. 3A). Direct sequence analysis of the smaller transcript revealed that it lacked the codons for the transmembrane domain of Fas (Fig. 3B). This mRNA variant was described as coding for soluble Fas (sFas) (Cascino et al, 1995; Cheng et al, 1994). However, we could not detect any sFas protein in cell culture supernatants of malignant B cells by immunoprecipita-tion and Western blotting (Fig. 3C).

Malignant B Cells Show Mutations in Fas Transcripts but No LOH in the Locus of the Fas Gene

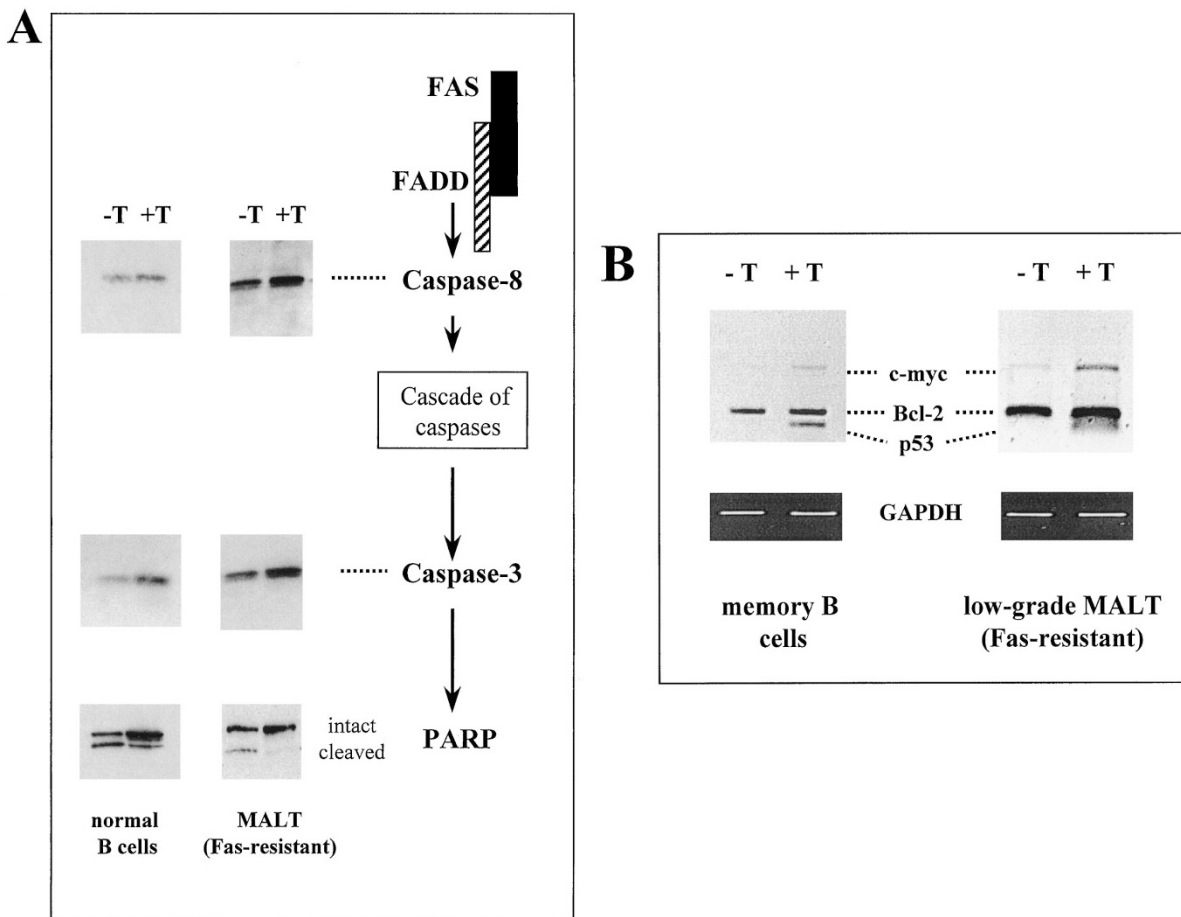In further investigations we screened the Fas molecule for mutations. The coding region of Fas was amplified

Figure 2.

A, Caspases-3 and -8 are detected by Western blotting in Fas-resistant malignant B cells (Case 6) as well as in normal B cells. However, cleavage of poly-ADP ribose polymerase (PARP) is abrogated by T cell influence only in the malignant B cells. Malignant B cells were either cultured for 24 hours in medium alone (−T) or cocultured with activated T cells (+T) that were removed afterward. B, The expression pattern of c-myc, Bcl-2, and p53 shows no difference between Fas-resistant malignant B cells and normal memory B cells as determined by reverse transcriptase polymerase chain reaction (RT-PCR). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
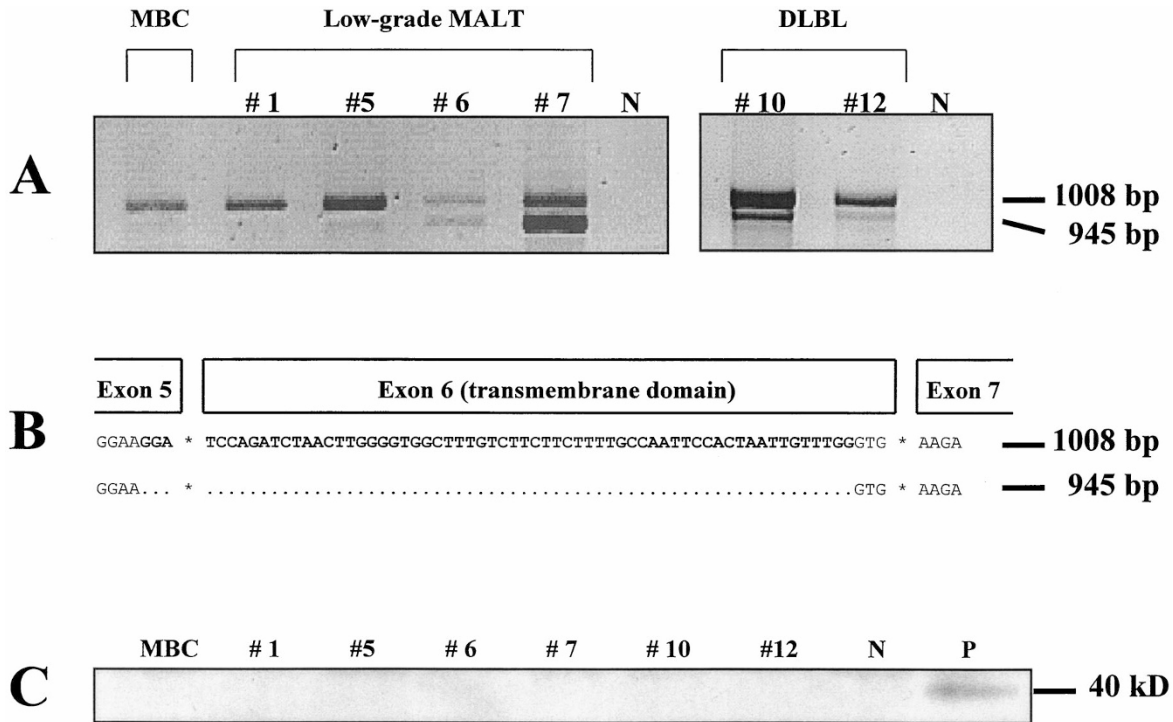
Figure 3.
Malignant B cells produce soluble Fas (sFas) mRNA, but no protein. A, RT-PCR of the Fas transcript reveals a variant which lacks 63 bp. This variant is found in Fas-resistant MALT-type lymphoma (Cases 5 and 6) and DLBL only. MBC, normal memory B cells as control; N, negative control. B, Direct sequence analysis shows that the smaller Fas transcript is a splicing variant lacking exon 6, the transmembrane domain. C, Immunoprecipitation of sFas from cell culture supernatants demonstrates no detectable sFas protein in any of the cases investigated. N, negative control; P, positive control (immunoprecipitation of 100 ng of recombinant human sFas).

using RT-PCR, cloned, and sequenced. Apart from various randomized mutations occurring in only one of the clones, we also found point mutations in two or more independent clones investigated (Fig. 4). For the analysis we focused on the latter and found 10 different point mutations in four MALT-type lymphomas (Cases 1, 5, 6, and 7) and one DLBL (Case 9, Table 1). Case 1 showed one mutation, Cases 5 and 7 two mutations each, and Case 6 four mutations, whereas the DLBL Case 9 possessed three mutations. In particular, the 10 mutations consisted of seven transitions, two transversions, and one 1-bp insertion. Four of the 10-point mutations caused a change in the predicted amino acid sequence at codons 23, 50, 172, and 229, respectively, whereas four mutations affected the third position of the codons 27, 237, 255, and 291 (silent mutations, Table 1), thus having no relevance for the amino acid sequence. One mutation introduced a premature termination signal after codon 152. The 1-bp insertion caused a frame shift after codon 284, resulting in 19 new amino acids and premature termination thereafter, so that the predicted protein would lack 16 amino acids.

To investigate loss of heterozygosity (LOH) in the Fas gene locus, we used two flanking microsatellite markers, D10S541 and D10S1739. These two markers were informative in 77% and 66% of cases. However, in none of the patients investigated was any mono- or biallelic LOH found (data not shown).

Figure 4.
Nucleotide sequence alignment of cloned Fas transcripts from malignant B cells of Cases 6 and 7. In the two patients, codon 23 is affected by the same mutation in multiple clones, whereas the codons 43 and 57 show randomized mutations only in one clone each. The normal Fas sequence and the numbering of codons is according to Itoh et al (1991).

Discussion

Recently, evidence emerged for a link between autoimmunity and non-Hodgkin's lymphomas (NHL),

Table 1. Fas Mutations in Malignant B Cells from MALT-Type Lymphoma

Codon[a]	Mutation (Amino acid change)	Mutated clones/normal clones	Case No.	Resistance to apoptosis
23	Arg)A → G	4/12	6	yes
	(Lys → Arg)	3/21	7	yes
27	A → G (silent)	3/17	5	
50	G → A Gly → Ser	2/18	5	yes
152	G → T (Glu → STOP)	2/4	9	yes
172	T → C Val → Ala	2/14	6	yes
229	T → C Val → Ala	2/4	9	yes
237	T → C (silent)	2/35	6	
255	A → T (silent)	2/4	9	
284	1-bp insertion (A)	3/19	1	no
		2/34	7	yes
291	A → G (silent)	3/21	6	

Lys, lysine; Arg, arginine; Gly, glycine; Ser, serine; Glu, glutamic acid; STOP, STOP-CODON; Val, valine; Ala, alanine.
[a] Numbering of codons is according to Itoh et al (1991).

which may be based on disturbances in Fas-mediated apoptosis (Plumas et al, 1998). MALT-type lymphomas of the thyroid gland, salivary gland, or the stomach show a strong association with organotropic autoimmune diseases that precede tumor development as initial lesion. Previous studies of a large panel of B cell lymphomas demonstrated the presence of Fas mutations in some classical Hodgkin's cases (Müschen et al, 2000) and in different B cell NHLs, reporting Fas mutations in 16 of 150 (11%) cases, with the highest incidence (60%) among extranodal lymphoma (Gronbaek et al, 1998).

Therefore, we focused our study on lymphomas of MALT-type, the largest group within extranodal NHLs. We found 10 previously undescribed point mutations in Fas transcripts from malignant B cells from four of five MALT-type lymphomas and from one of three DLBLs investigated. Because we have exclusively considered mutations that occurred in at least two independent Fas cDNA clones of the same patient or in different patients, we can rule out PCR artifacts. Six of 10 mutations lead to amino acid changes in the Fas protein and might thus have a potential functional relevance. Interestingly, five out of these mutations were found in malignant B cells that were resistant to T cell-induced, Fas-mediated apoptosis.

The Fas mutations described herein are located both in the extra- and the intracellular domain of Fas and might therefore interfere with FasL binding (eg, mutation in codons 23 and 50) and Fas signaling

(eg, mutation in codon 229). The 1-bp insertion (+A) after codon 284, found in a Fas-resistant and a Fas-sensitive case, more likely represents a DNA polymerase error resulting from a slippage mechanism in a run of identical nucleotides (Tindall and Kunkel, 1988) than a true mutation that would result in a truncated protein.

Although we could not detect Fas mutations in all cases resistant to apoptosis so far, our sequencing data, in combination with our functional data, support the idea of an association between Fas mutations and resistance to apoptosis. Because it was shown recently that mutated Fas proteins impair apoptosis in a dominant negative way (Siegel et al, 2000), this would also explain our finding that for each mutation only a minority of the Fas transcripts was affected.

However, factors other than Fas mutations could cause impairment of Fas-mediated apoptosis. One mechanism of protection could lie in the reduced Fas expression that was found in vivo in malignant cells of hematologic and nonhematologic origin (Owen-Schaub et al, 1993) and in T cell leukemia cell lines in vitro (Martinez-Lorenzo et al, 1998). This mechanism appears not to be operative in MALT-type lymphoma, because all DLBLs that are resistant to apoptosis showed, not reduced, but rather enhanced constitutive Fas expression when compared with normal B cells. Furthermore, those malignant B cells from MALT-type lymphoma with the lowest Fas expression were sensitive to apoptosis.

A second mechanism of resistance to apoptosis described so far, is the production of soluble Fas (sFas) protein that antagonizes the normal function of Fas by the binding of FasL (Cheng et al, 1994). sFas is generated by alternative splicing of exon 6 coding for the transmembrane domain and was found in phytohemagglutinin-activated normal human lymphocytes (Cascino et al, 1995) from patients with autoimmune disease (Cheng et al, 1994) and in nonhematopoietic and lymphatic malignancies (Knipping et al, 1995; Midis et al, 1996; Yufu et al, 1998). Recently, sFas protein was found in the serum of some patients with MALT-type lymphoma (Doi et al, 1999), and it was speculated that it originates in the tumor cells. However, although we found mRNA for sFas in the purified malignant B cells from MALT-type lymphoma, this mRNA was not translated into protein, because sFas was completely absent in the tumor cells supernatants.

Resistance to apoptosis could also be due to impairment of the signaling cascade downstream of Fas, because it was shown recently in caspase-3 or caspase-8 knockout-mice (Varfolomeev et al, 1998; Woo et al, 1999) and in a human T cell line defective in caspase-3 (Martinez-Lorenzo et al, 1998). However, we found caspase-3 and caspase-8 proteins and basic levels of PARP cleavage in MALT-type lymphomas, indicating that the signaling cascade is intact and facilitates spontaneous apoptosis without T cell stimuli. This baseline apoptosis was abrogated after coculture with activated T cells, indicating cosignaling effects resulting from CD40-ligation (An and Knox,

1996). Furthermore, the missing inducibility of caspase expression or processing under T cell influence is an additional clue to an impaired Fas molecule not transducing an apoptotic signal.

Fas mutations resulting in loss of function imply that Fas may act as a tumor suppressor gene. This hypothesis is supported by recent findings of genomic instability as detected by LOH in the Fas promotor region in solid tumors (Lee et al, 1999a, 1999b; Shin et al, 1999), but is in contrast to results with MALT-type lymphoma, because we could not find LOH in any of the cases investigated. However, hypermutation and ongoing mutations of MALT-type lymphoma antigen receptors support the notion that clonal growth of malignant B cells occurred during or after the germinal center development (Hallas et al, 1998; Qin et al, 1997). Therefore, it is possible that various Fas mutations, some of them resulting in loss of function and thus inhibition of apoptosis, may arise in mature B cells during variable, diverse, and joining gene segments (V[D]J) recombination and other diversification processes in the course of immune responses, as was shown recently for the bcl-6 gene (Peng et al, 1999; Shen et al, 1998). This would characterize the Fas mutations as an early event in MALT-type lymphomagenesis. The concept of dysregulated apoptosis in MALT-type lymphoma was supported by parallel examinations in the identification of specific chromosomal aberrations in these tumors. In particular, a rare t(1;14)(p22;q32) involves the apoptosis regulatory gene bcl-10 (Willis et al, 1999), and the more frequent translocation in MALT-type lymphomas t(11;18)(q21;q21) (Ott et al, 1997; Rosenwald et al, 1999) involves the apoptosis inhibitor gene HIAP-1 (Dierlamm et al, 1999). However, we found the t(11;18) in two of three MALT-type lymphomas resistant to apoptosis and also in two of four cases sensitive to apoptosis. Furthermore, although HIAP-1 was described to inhibit caspases directly (Roy et al, 1997), we found that caspases-3 and -8 were processed irrespective of the t(11;18). This indicates that the fusion protein HIAP-1/malt-type lymphoma-associated translocation (MLT) resulting

from the t(11;18) may interfere with other apoptosis pathways. Additionally, the presence of t(11;18) may not have an influence on the Fas mutation frequency, because there was no obvious difference between t(11;18)-positive (2.35×10^{-4}/bp) and t(11;18)-negative MALT-type lymphomas (2.82×10^{-4}/bp) compared with DLBL (10.7×10^{-4}/bp).

Therefore our results characterize the t(11;18) as an additional event that may occur later in the course of MALT-type lymphomagenesis. Several events with similar consequences may join or interfere within MALT-type lymphomas, but ultimately they emphasize the principle of inhibition of apoptosis in MALT-type tumor pathogenesis.

Materials and Methods

Patients

Tumor specimens from patients with malignant lymphomas ($n = 11$) were obtained fresh from the tissues after surgical removal. Seven cases were diagnosed as MALT-type lymphomas and four cases as gastric DLBLs according to the revised European-American lymphoma (REAL) classification (Harris et al, 1994) (Table 2). The diagnosis was established by morphological and immunophenotypic analysis of fresh-frozen and paraffin-embedded sections as described (Greiner and Müller-Hermelink, 1996). Reactive normal tonsillar tissues from routine tonsillectomy were used as a control.

The presence of the t(11;18)(q21;q21) in malignant B cells was determined by applying a fluorescence in situ hybridization (FISH) assay that was recently developed to detect the chromosomal breakage in 11q21 in interphase cells (Rosenwald et al, 1999).

Cell Preparation and Culture

Small pieces of tumor tissue were pressed through sterile sieves. T and B cells from the resulting suspension were separated using magnetic beads coupled with antibodies (Dynal, Hamburg, Germany). T cells were isolated by using α-CD3 beads and Detach-a-

Table 2. Characteristics of the Investigated Patients

Case No.	Diagnosis/staging[a]	Localization	Sex	Age	Apoptosis	t(11; 18)[b]
1	MALT/—	Parotis	F	66	Sensitive	No
2	MALT/—	Parotis	F	61	Sensitive	No
3	MALT/E I2	Stomach	M	59	Sensitive	Yes
4	MALT/—	Lung	—	—	Sensitive	Yes
5	MALT/—	Thyroid	M	70	Resistant	Yes
6	MALT/—	Lung	—	—	Resistant	Yes
7	MALT/—	Stomach	F	69	Resistant	No
8	DLBL/E II1	Stomach	M	74	Resistant	No
9	DLBL/E I2	Stomach	M	31	Resistant	No
10	DLBL/—	Stomach	M	61	Resistant	No
11	DLBL/E III	Stomach	M	64	Resistant	No

[a] Staging was done according to Musshoff modified by Radaszkiewicz et al (1992).
[b] Fluorescence in-situ hybridization (FISH) data according to Rosenwald et al (1999).
—, data not available; MALT, mucosa-associated lymphoid tissue; DLBL, diffuse large B cell lymphoma.

beads thereafter (Dynal). B cells were depleted negatively by removing T cells and macrophages with α-CD2 and α-CD14 beads, respectively. Malignant B cells were negatively purified from nonmalignant bystander B cells by using magnetic beads coupled with antibodies against the particular immunoglobulin light and heavy chains not expressed by the lymphoma as described in detail recently (Greiner et al, 1997). Normal memory B cells (CD19$^+$, IgM$^+$, CD38$^-$, IgD$^-$) that were negatively depleted from human tonsils served as controls. Cells were cultured in flat-bottom 24-well plates in a final volume of 1 ml at 37° C and 7% CO_2 in RPMI 1640 supplemented with gentamycin (0.1 mg/ml) and 10% FCS.

To investigate the interaction of malignant B cells with activated T cells, we established an experimental approach of an in vitro B/T cell coculture. Heterologous T cells were activated for 6 hours by α-CD3 stimulation using the monoclonal antibody 4B5 (Boehringer, Mannheim, Germany) presented by a murine fibroblast cell line that was transfected with human FcγRII (Banchereau and Rousset, 1991). Thereafter, those T cells were cocultured in five-fold excess for 24 hours with malignant B cells from MALT-type lymphoma and DLBL, respectively. Before performing RNA and protein extraction from B cells, the T cells were removed immunomagnetically as described above. In general, each cell separation was checked on FACS, and cells were processed further if purity was above 95% in each setting. Epstein-Barr virus (EBV) cell lines from purified lymphoma B cells were generated (Cases 5 and 7) and served as independent controls for Fas sequencing.

Preparation of RNA and cDNA Synthesis

Total RNA was extracted either from 2×10^5 highly purified malignant or normal memory B cells using TRIzol reagent (Life Technologies, Paisley, United Kingdom) according to the manufacturer's instructions. First-strand cDNA synthesis was carried out with 1 μg of total RNA as described in detail elsewhere (Knörr et al, 1999). The quality and amount of cDNA was estimated by PCR using glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific primers. PCR reactions were performed in the GeneAmp PCR system 2400 (Perkin-Elmer Cetus, Emeryville, California) in a final volume of 25 μl containing 1 μl of cDNA, 2.5 μl of $\times$10 PCR buffer (Amersham Pharmacia,

Freiburg, Germany), 1.5 μml of 25 mM $MgCl_2$, 0.5 μl of 10 mM dNTPs, 0.5 μl of 20 pmol/μl of each primer and 0.5 U of cloned *Taq* DNA polymerase (Amersham Pharmacia). Amplifications consisted of 20 cycles of denaturation at 94° C for 30 seconds, annealing of primers at 60° C for 30 seconds, extension of primers at 72° C for 45 seconds, and a final polymerization step of 4 minutes at 72° C. PCR products were analyzed on a 2% agarose gel (Sigma, Deisenhofen, Germany), stained with ethidium bromide, and photographed under UV light.

RT-PCR of Apoptosis-Relevant Genes

For investigating the expression of apoptosis-relevant genes, two multiplex RT-PCR kits (hAPO-1 and hAPO-2; Maxim Biotech, San Francisco, California) were used. The hAPO-1 kit was designed for simultaneous analysis of caspase-1, c-myc, bcl-2, and p53, and the hAPO-2 kit for caspase-3, bcl-xL, bcl-2, bcl-xS, and bax. According to the manufacturer's instructions, the amplifications were as follows: 2 cycles (96° C for 1 minute, 60° C for 4 minutes) and 28 cycles (94° C for 1 minute, 60° C for 2, 5 minutes) followed by a final polymerization step of 10 minutes at 70° C.

Cloning and Sequencing of Fas

The entire coding region of Fas (1008 bp, accession no. M67454) was amplified in two steps by RT-PCR using the primer pairs CD95-I-For/CD95-GR46-Rev and CD95-GR19-For/CD95-III-Rev (Table 3). For the amplification we used a recombinant *Taq* DNA Polymerase (Amersham Pharmacia) that showed in our experimental system an error rate of 0.17% (C. Knörr, University of Würzburg, personal communication, 2000). The amplification consisted of initial denaturation at 94° C for 60 seconds followed by 33 cycles of denaturation at 94° C for 30 seconds, annealing of primers at 61° C for 30 seconds, extension of primers at 72° C for 60 seconds, and a final polymerization step at 72° C for 7 minutes. The PCR products were cloned into pCRscript (Stratagene, Heidelberg, Germany), which was used to transform ultracompetent *Escherichia coli* (Stratagene). White colonies were picked and grown overnight in 3 ml of Luria-Bertani (LB) medium. The double-stranded DNA template from the colonies was sequenced by the chain-

Table 3. Primers Used for RT-PCR

Primer	Sequence
GAPDH For	5′-GTG GAA GGA CTC ATG ACC ACA GTC-3′
GAPDH Rev	5′-CAT GTG GGC CAT GAG GTC CAC CAC-3′
CD95-I-For[a]	5′-ATGCTGGGCATCTGGACCCT-3′
CD95-GR46-Rev[b]	5′-CATTGACACCATTCTTTCGAA-3′
CD95-GR19-For[b]	5′-AACATGCAGAAAGCACAGAAA-3′
CD95-III-Rev[a]	5′-TCTAGACCAAGCTTTGGATTTC-3′

[a] Primers according to Fiucci and Ruberti; (1994).
[b] Primers according to Cascino et al (1996).

terminating method (Sanger et al, 1977) using the *Taq*-dye-deoxy termination cycle sequencing kit (Applied Biosystems, Weiterstadt, Germany). Sequencing was performed on the ABI 373A automatic sequencer (Applied Biosystems).

Detection of Loss of Heterozygosity

Two polymorphic dinucleotide repeats flanking the Fas gene, D10S541 and D10S1739, in a distance of 1.2 centimorgans (cM) were chosen from the Genome Data Base (GDB Human Genome Database, 2001) and primers synthesized according to the sequences provided. The 5' ends of the forward primers were labeled with fluorescent dyes HEX and FAM, respectively (PE Biosystems, Weiterstadt, Germany). PCRs were performed as described in detail elsewhere (Starostik et al, 2000). PCR products were electrophoresed on a 4.5% polyacrylamide gel containing 45% urea for 2–3 hours at 150 W on the ABI 377 Sequencer (Applied Biosystems).

Protein Extraction and Immunoblotting

Total protein was extracted from 1×10^6 highly purified malignant or normal memory B cells as described (Laemmli, 1970). Proteins were resolved in SDS-PAGE, transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany), and incubated with primary rabbit antibody (1 μg/ml) for 1 hour at room temperature followed by a secondary α-rabbit antibody linked to horseradish peroxidase (Amersham, Braunschweig, Germany). Signals were visualized by enhanced chemoluminescence (ECL, Amersham) according to the manufacturer's instructions. The following primary antibodies were used (all from Santa Cruz, Heidelberg, Germany): α-caspase-3 (N-19), α-caspase-8 (T-16), and α-Poly-ADP Ribose Polymerase (PARP, H-250).

Immunoprecipitation

Cell culture supernatant (800–1000 μl) of normal and malignant B cells was incubated for 1 hour at 4° C with 1 μg of α-sFas antibody (G 254–274; Pharmingen, Hamburg, Germany). As a positive control, 100 ng of recombinant human sFas (Pharmingen) were diluted in 1000 μl cell culture medium and treated accordingly. Then Protein A/G-agarose (Santa Cruz, Heidelberg, Germany) was added to the samples and the same incubation step was repeated. The proteins were precipitated by centrifugation, washed, resolved in Laemmli buffer without reducing agents such as dithiothreitol (DTT), and subjected to SDS-PAGE. Immunoblotting was carried out with the same antibody (G 254–274) that was used for precipitation.

Flow Cytometry

Three-color flow cytometric analysis was performed with a FACScan (Becton Dickinson, Mountain View, California) using the program LYSIS II for data acquisition and analysis. For investigating B cells, the instrument was set up by locating the B cells of an unstained sample in a standard position in the display of forward and sideward light scattering by adjustment of the light-scattering detectors. Thereafter samples stained with fluorescein-isothiocyanate (FITC)-, phycoerythrin (PE)-, and Quantum Red (QR)-conjugated CD19 antibodies were measured. To avoid spectral overlappings between the three fluorescence channels, the fluorescence detectors were adjusted accordingly.

The following antibodies were used: α-CD19-FITC (F 768; Dako, Hamburg, Germany), α-CD19-PE (M 740, Dako), α-CD19-QR (MHCD 1906; MEDAC, Hamburg, Germany), and α-APO-1-PE (kind gift from P. Krammer, Deutsches Krebsforschungszentrum, Heidelberg). A corresponding isotype control was also measured for each antibody: IgG1-FITC (F-6397; Sigma Chemical Co., St. Louis, Missouri), IgG1-PE (P4685, Sigma), and IgG1-QR (MG106TC, MEDAC).

To measure apoptosis, the DNA-binding reagent, 7-amino actinomycin D (7-AAD), was used as described elsewhere (Philpott et al, 1996). In samples of B/T cell coculture, only cells positive for CD19 (B cells) were analyzed for 7-AAD binding and Fas expression. At least 40,000 cells were recorded for a FACS analysis. [Editor: Author has cited the following references in tables: Cascino et al, 1996; Fiucci and Ruberti, 1994; Radaszkiewicz et al, 1992.]

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