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Advances in Brief

Somatic Mutations of the *CD95* Gene in Hodgkin and Reed-Sternberg Cells[1]

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Abstract

Hodgkin and Reed-Sternberg (H/RS) cells in classical Hodgkin's disease (cHD) are thought to be derived from preapoptotic germinal center B cells. However, little is known about the transforming events rescuing the precursor of the H/RS cells from apoptosis. Given the importance of CD95 (Apo-1/Fas)-mediated apoptosis for negative selection within the germinal center, single micromanipulated H/RS cells from 10 cases of cHD were analyzed for somatic mutations within the *CD95* gene. Three clonal mutations within the 5′ regions were amplified from single H/RS cells in one case. From H/RS cells of another case, two mutations within the last exon coding for the death domain were detected. About half of these H/RS cells carried a monoallelic stop-codon; the remaining tumor cells harbored a monoallelic replacement mutation. Both mutations likely impair *CD95* function. Because all these H/RS cells also bear clonal mutations inactivating the *IκBα* gene, the *IκBα* mutations occurred earlier than those of the *CD95* gene in the sequence of transforming events leading to cHD. In conclusion, somatic mutations of the *CD95* gene occur in a fraction of cHD cases and may favor the escape of the precursor of the H/RS clone from apoptosis.

Introduction

H/RS[3] cells have recently been demonstrated to be derived from GC B cells (1). In many cases of classical cHD, the H/RS cells have lost their capacity to express a functional B-cell receptor due to destructive somatic mutations of the rearranged immunoglobulin genes (2–4). As GC B cells are destined to die by apoptosis within the GC unless they are positively selected for expression of a B-cell receptor with high affinity to antigen (5), the hypothesis was developed that cHD may represent the outgrowth of a preapoptotic GC B cell (1–4). It remains an open question, however, how the precursor of the H/RS cell clone escaped apoptosis during the GC reaction.

In the B lineage, CD95 (Apo-1/Fas) is expressed specifically at the GC stage of differentiation (6). CD95-mediated apoptosis was proposed to represent an important mechanism for negative selection of B cells within the GC (6, 7). Deleterious mutations of the *CD95* gene should therefore confer resistance of GC B cells to a major pathway of apoptosis in the GC. Indeed, in *CD95*-deficient *lpr* mice, autoreactive B cells can escape negative selection (8), resulting in lymphadenopathy, enlargement of liver and spleen, and propensity to auto-

immunity (8) and B-cell lymphoma (9). Germ-line mutations of the *CD95* gene leading to autoimmune lymphoproliferative syndrome and predisposing to B-cell lymphoma and other malignancies occur in humans as well (10, 11). Notably, some patients carrying deleterious mutations of the *CD95* gene in their germ-line developed lymphocyte-predominant HD (12) or cHD (11). Somatic mutations impairing the transduction of the apoptosis signal were observed in a number of lymphoid malignancies (13–15). In lymphomas derived from antigen-experienced B cells, mutations of the *CD95* gene may have been acquired during the GC reaction and represent a side effect of somatic hypermutation acting outside the immunoglobulin loci.[4] However, T cell-derived malignancies (15) and solid tumors (16–18) also were found to harbor somatic mutations of the *CD95* gene. Deleterious mutations of exon 9, coding for the DD, act in a dominant negative way impairing *CD95* function as a whole (10). The dominant negative effect of monoallelic mutations within the DD is likely attributable to the trimerization of the CD95 receptor on the cell surface. The DD is a highly conserved region that is required and sufficient for the transduction of the death signal (19). Given the functional importance of this region, it is not surprising that about 60% of somatic mutations in lymphoid or solid tumors involve this region (18).

It was recently shown that several HD-derived cell lines, although expressing *CD95*, are resistant to CD95-mediated apoptosis (20). To clarify whether impairment of CD95-mediated apoptosis is due to somatic mutations of the *CD95* gene, which may thus contribute to the persistence of the preapoptotic GC B cells developing toward cHD, single micromanipulated H/RS cells from 10 cases of cHD were analyzed for such mutations.

Materials and Methods

Case Description. Information on the 10 cases of cHD from which single micromanipulated H/RS cells were analyzed is given in Table 1. Cases I–III, IV–VIII, and X have been studied previously (Refs. 4, 21, and 3, respectively).

Immunostaining. For immunostaining, 7-μm-thick frozen tissue sections were stained using antibodies against CD30, CD20, anaplastic lymphoma kinase-1 (Dako), CD15 (Becton Dickinson, Mountain View, CA), and CD3 (Ortho Diagnostic Systems, Raritan, NJ).

Micromanipulation and Single-Cell PCR for the *CD95* Gene. Stained cells were mobilized and aspirated with the help of a micropipette fixed to a hydraulic micromanipulator. Buffer covering the sections was aspirated as negative controls for PCR analysis.

For all micromanipulated H/RS cells, a whole genome preamplification step (22) was performed. The H/RS cells were first analyzed for immunoglobulin-heavy chain and immunoglobulin κ and λ-light chain as well as TCRβ gene rearrangements. In nine cases, the *CD95* gene was amplified only from preamplification reactions that already gave rise to the H/RS cell-specific immunoglobulin or to *TCRβ* gene rearrangements. In one case (case V; Table 2), no preamplification reactions were available, and H/RS cells were subjected directly to two rounds of PCR amplification of the *CD95* gene. Aliquots of 4 μl from these reactions were then subjected to two rounds of seminested PCR amplification as described previously. Briefly, rearranged V_H-, Vκ-, and

[4] M. Müschen *et al.*, submitted for publication.

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Table 1 *Case description of patients with cHD*

Case	Age	Sex	Presentation	LN biopsy site	HD subtype	CD30	CD15	EBV[a]
I	31	Male	First	Abdominal	MC[b]	+	+	+
II	26	Male	First	Cervical	MC	+	+	+
III	51	Male	First	Inguinal	NS[c]	+	+	−
IV	12	Female	First	Submandibular	NS	+	+	+
V	20	Male	First	Axillar	NS	+	+	−
VI	35	Male	First	Supraclavicular	NS	+	+	−
VII	29	Male	Relapse	Cervical	MC	+	+	+
VIII	36	Male	First	Abdominal	MC	+	+	+
IX	68	Male	First	Cervical	MC	+	+	+
X	19	Male	First	Cervical	MC	+	+	+

[a] EBV-infection was assessed either by amplification of a specific fragment of the *EBNA1* gene from single H/RS cells (cases I, II, III, and IX) or by *in situ* hybridization for EBV-encoded small RNA and staining for LMP1 in the remaining cases.

[b] MC, mixed cellularity; NS, nodular sclerosis.

*V*λ-genes were amplified using family-specific framework region I *V*-gene primers and two sets of J$_H$-, Jκ- and Jλ-primers in a seminested approach (3). TCRβ VDJ, and TCRβ DJ gene rearrangements were amplified as described previously (4). PCR products were gel-purified and directly sequenced using the BigDye Terminator cycle sequencing kit and an automated sequencer (ABI 377; Applied Biosystems, Germany). As depicted in Fig. 1, two regions of the *CD95* gene were analyzed by single-cell PCR. Exon IX coding for the DD (Fig. 1) was amplified from H/RS cells of all 10 cases of cHD 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. For cases I, II, III, IX, and X, a fragment encompassing the 5′ untranslated and coding regions of exon I and the 5′ part of the first intron (Fig. 1) was amplified from multiple H/RS cells using 5′-ACC ACCGGG 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 GGG CTG CAG-3′ as external reverse, and 5′-CGG AGC GGA CCT TTG GCT-3′ as internal reverse primers.

Determination of EBV Infection. EBV infection of H/RS cells was examined by *in situ* hybridization for noncoding EBV-encoded small RNAs, by amplification of a fragment of the *EBV nuclear antigen-1* gene from single micromanipulated H/RS cells (4), or by immunostaining for latent membrane protein 1.

Results

Single H/RS cells from 10 cases of cHD (Table 1) were micromanipulated and subjected to whole genome preamplification. With one exception, for further analysis only whole genome preamplification reactions of H/RS cells were selected from which the immunoglobulin or TCRβ gene rearrangement defining the H/RS tumor clone could be amplified (Table 2; see "Materials and Methods"). The last exon of the *CD95* gene coding for the DD was amplified from multiple H/RS cells of all 10 cases. From H/RS cells of five cases, a 750-bp fragment of the *CD95* gene encompassing 5′untranslated and coding regions of

Table 2 *Sequence analysis of 5′ regions and the DD of the CD95 gene amplified from single micromanipulated H/RS cells*

| Case	Markers of clonality	Region	PCR positive[a]		Mutations in H/RS cells
			H/RS cells	Buffer controls	
I	*Ig V$_H$2*[b]	5′R[c]	6/6	0/2	3 mutations
	Ig D$_H$3[b]				−92A[d]→G
					−92A: 2 cells; −92G: 4 cells
					+108C→T
					+108C: 2 cells; 108T: 4 cells
					+162C→T
					+162C: 2 cells; 162T: 4 cells
		Exon 9	4/5	0/4	0
II	*Ig V$_H$1*[b]	5′R[c]	2/4	0/2	0
	Ig V$_H$3[b]	Exon 9	5/7	0/4	0
	Ig Vκ1[b]				
III	*TCR Vβ7*[b]	5′R[d]	2/4	0/2	0
	TCR Dβ1[b]	Exon 9	5/8	0/4	0
IV	*Ig V$_H$1*	Exon 9	12/12	0/6	2 mutations
	Ig Vκ1				Codon 295[d]
	Ig Vκ3				GAA→TAA (Glu→Stop)
	Ig Vκ4				1162G: 5 cells; 1162K: 7 cells
	2 Ig Vλ1				Codon 282[d]
					ATT→GTT (Ile→Val)
					1122A: 7 cells; 1122R: 5 cells
V	none[e]	Exon 9	4/7	0/3	0
VI	*Ig Vκ1*	Exon 9	5/6	0/3	0
	Ig Vκ2				
VII	*Ig V$_H$3*	Exon 9	4/5	0/3	0
VIII	*2 Ig V$_H$4*	Exon 9	4/6	0/3	0
IX	*Ig V$_H$1*	5′R[c]	2/4	0/2	0
	Ig Vκ2				
	Ig Vκ3	Exon 9	5/8	0/4	0
X	*Ig V$_H$3*[b]	5′R[c]	2/5	0/2	0
		Exon 9	3/5	0/4	0

[a] All PCR products were sequenced.

[b] Clonal Ig- and TCRβ-gene rearrangements have been published previously (GenBank accession no. Z77316-31, AJ243643-8, and AJ251000-2).

[c] Two novel germ-line polymorphisms of 5′ noncoding regions of the CD95 (5′R) have been identified in cases I, II, III, IX, and X. Both polymorphic alleles have been amplified repeatedly.

[d] Numbering of nucleotide positions refers to the ATG as +1, codon numbering is according to Itoh *et al.,* 1991 (GenBank accession no. M67454).

[e] From other H/RS cells of this case, a clonal mutation of the *IκBα* gene was amplified (GenBank accession no. AJ249283-9, and AJ249294-5).

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Fig. 1. Organization of the *CD95* gene and PCR strategy. The organization of the *CD95* gene (comprising exons I to IX) is depicted (not to scale). From H/RS cells of five primary cases of cHD a 750 bp fragment encompassing 5′ untranslated (5′UTR, *gray boxes*) and coding (*black boxes*) regions of the first exon and a p53 responsive intronic enhancer was amplified. From H/RS cells of ten primary cases of cHD a 440 bp fragment containing the region of exon IX coding for the death domain of *CD95* was amplified. Mismatches to known germ-line sequences are depicted by vertical lines (five somatic mutations with *arrowheads*, two novel germ-line polymorphisms without).
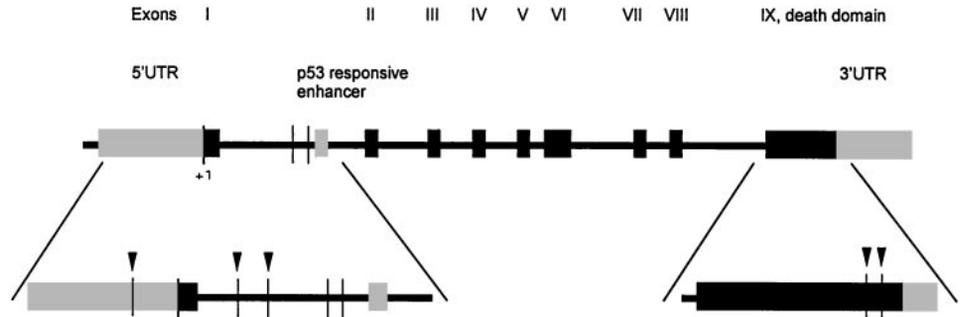
exon I and a p53-responsive enhancer within the first intron was analyzed, here collectively termed 5′R (Fig. 1; Table 2).

In one of 10 cases of cHD, the H/RS cells harbored monoallelic somatic mutations of the last exon. From all 12 H/RS cells analyzed from that case, a somatically mutated *CD95* gene was coamplified with a wt allele (Fig. 2*A*). Unexpectedly, the mutations are not shared between all H/RS cells; five of the cells carry a mutation leading to an amino acid replacement at codon 282 (Ile→Val). From seven H/RS cells of the same case, a mutation that truncates the DD through a translational stop at codon 295 was amplified. Given that all truncating or replacement mutations within the highly conserved DD described thus far act in a dominant negative way and are related to a clinical phenotype (10, 11, 18), these two mutations are also likely to interfere with CD95-mediated apoptosis.

These mutations either define two distinct subclones (Fig. 2) or indicate the presence of a third allele of the *CD95* gene in the H/RS cells. The latter possibility, however, is unlikely, as both mutations were not concomitantly amplified from any of the H/RS cells; whereas PCR amplification from all 12 H/RS cells gave rise to one of the mutant alleles together with a wt allele. The two H/RS cell populations defined by distinct mutations of the *CD95* gene belong to the same clone, as they harbored both the same immunoglobulin gene rearrangements (Table 2, Fig. 2*A*) and biallelic clonal mutations of the *IκBα* gene (21).

The 5′R of the *CD95* gene from H/RS cells of five cases of cHD also were amplified and sequenced. Within these regions, two novel intronic germ-line polymorphisms of the *CD95* gene were identified (Fig. 1, Table 2)[4] and confirmed by sequencing from whole-tissue DNA or single micromanipulated CD3^{+} T cells from tissue sections of the same case. Using the two polymorphisms as allelic markers, none of four informative cases showed allelic loss of the *CD95* gene (*i.e.*, each polymorphic allele could be amplified at least once). From the H/RS cells of one case, three clonal mutations within the 5′R were amplified. The three mutations in this case were found either concomitantly (four cells) or only the wt allele was amplified (two cells; Table 2), indicating that the three mutations are present on one allele. These three mutations are unlikely to silence *CD95* function, as only noncoding regions were involved. A p53-responsive intronic enhancer that is required for CD95 transcription (23) is also situated in this region but was not mutated.

Extending a previous analysis on mutations of the *CD95* gene in cHD-derived cell lines (20), the DD and 5′R were analyzed in the putative cHD cell lines L1236, L428, L540, L591, DEV, HDLM-2, and KM-H2, and no mutation was found.

Discussion

As opposed to the malignant cells of other lymphomas, H/RS cells are thought to be derived from a preapoptotic GC B cell. Therefore, defects in the transduction of proapoptotic signals or in the execution of the apoptosis program may be particularly important for the development of cHD. In about 50% of cases of cHD, EBV infection of the H/RS cells and expression of the EBV-encoded latent membrane proteins 1 and -2a may play a role in the rescue of the H/RS cell precursors from apoptosis (reviewed in Ref. 1). In a search for genetic defects in H/RS cells, *p53* mutations (24) and *bcl-2* translocations (25) were investigated in cHD at the single-cell level; however, no such events were found. Recently, deleterious mutations of the *IκBα* gene leading to constitutive nuclear NF-κB activity in H/RS cells were identified as the first genetic defect that may counteract the physiological susceptibility of the tumor precursor to apoptosis (21). However, *IκBα* inactivation due to deleterious somatic mutations was detected only in one of five primary cases and two of eight cHD-derived cell lines, indicating that other factors most likely contribute to apoptosis-resistance of H/RS cells.

As H/RS cells have been shown to coexpress *CD95* with its apoptosis-inducing ligand (26), defects in CD95 signaling may be critical for the survival of the tumor cells. Therefore, we studied 10
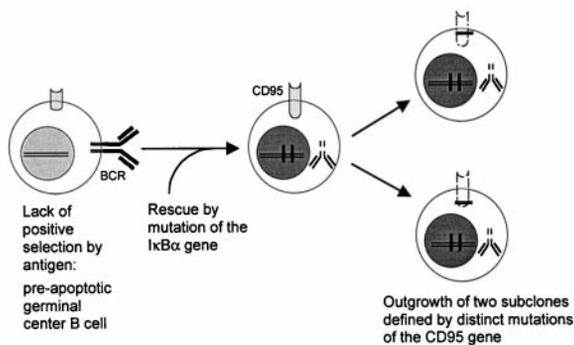

Fig. 2. Distinct mutations of the *CD95* gene define two subclones of the H/RS cells in case IV. *A*, three sequence variants obtained from 12 micromanipulated H/RS cells and whole tissue DNA (as a control for germ-line polymorphisms) are shown (*R*, A and G; *K*, G and T). *B*, based on the presence of clonal immunoglobulin gene rearrangements and clonal mutations of the *IκBα* gene together with two distinct mutations of the *CD95* gene defining two subclones the scenario of a possible sequence of transforming events leading to cHD is depicted.

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primary cases of cHD for *CD95* mutations. In 2 of the 10 cases, somatic mutations in the *CD95* gene were detected, one of which is likely to lack CD95 function as a result of destructive mutations. In the other case, three mutations were detected in the noncodoing 5′R whose impact on *CD95* function remains unclear.

For all 10 cases multiple H/RS cells were analyzed; thus most likely for all cases both *CD95* alleles were amplified. For the five cases that were informative for at least one polymorphic marker or which harbored somatic mutations, the presence of both alleles could indeed be verified. Thus, allelic loss of the *CD95* gene appears to occur rarely, if at all, in cHD. On the basis of the assumption of biallelic amplification of exon IX (440 bp) for 10 cases and 5′R (750 bp) for 5 cases, a rough estimate would yield a mutation frequency of 3.1×10^{-4}/bp for the *CD95* gene in H/RS cells. This frequency is not significantly different from that seen in normal GC B cells (2.2×10^{-4}/bp).[4] Therefore, the somatic mutations within the *CD95* gene in H/RS cells could merely reflect their GC B-cell nature. This particularly applies to somatic mutations within the 5′R, because about 15% of normal GC B cells carry *CD95* mutations in this region, which likely arise as a byproduct of the somatic hypermutation process.[4]

Somatic mutations within the DD frequently interfere with apoptosis-signaling, act in a dominant negative manner, and have been repeatedly observed in malignancy. Therefore, the two DD mutations amplified from H/RS cells in this study could well be involved in the malignant progression toward cHD. The H/RS cell population in the patient in question shares clonal somatically mutated immunoglobulin gene rearrangements and clonal mutations of the *IκBα* gene but is diversified by distinct mutations within the DD. The presence of clonal mutations of the *IκBα* gene together with two "subclonal" mutations within the DD of the *CD95* gene suggests that *IκBα* inactivation occurred earlier than the loss of *CD95* function in the sequence of transforming events leading to cHD (Fig. 2*B*). The finding that among a population of H/RS cells with clonal *IκBα* mutations two daughter cells with distinct *CD95* mutations established the H/RS tumor clone strongly suggests that the cells harboring these mutations were indeed positively selected and had a survival advantage. The consecutive silencing of *IκBα* and *CD95* in this case is reminiscent of the model by Fearon and Vogelstein (27), who identified a recurrent pattern of multistep carcinogenesis toward colorectal cancer. For future studies it will be interesting to clarify whether a recurrent sequence of transforming events can also be identified in cHD.

Taken together, somatic mutations in the DD of the *CD95* gene occur in a fraction of cases of cHD and may contribute to the pathogenesis of the lymphoma by impairing CD95-mediated apoptosis of the tumor cells.

Acknowledgments

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