J Mol Med (2000) 78:312–325
DOI 10.1007/s001090000112

REVIEW

M. Müschen · U. Warskulat · M.W. Beckmann

Defining CD95 as a tumor suppressor gene

Received: 21 December 1999 / Accepted: 18 April 2000 / Published online: 13 July 2000

Abstract The CD95 (Apo-1/Fas) receptor-ligand system is one of the key regulators of apoptosis and is particularly important for the maintenance of lymphocyte homeostasis. There is now broad evidence that susceptibility of tumor cells towards CD95-mediated apoptosis is largely reduced. In the human, germline and somatic mutations of the CD95 gene are associated with a high risk of both lymphoid and solid tumors. Based on these observations a new concept defining CD95 as a tumor suppressor gene is discussed. In addition to CD95, its natural ligand (CD95L) is also implicated in malignant progression. Compared to their nonmalignant counterparts, malignant cells frequently exhibit aberrant *de novo* expression of CD95L and are able to induce CD95L-mediated apoptosis in bystander cells. The role for neoplastic CD95L expression in local tissue destruction, invasion, and metastatic spread has been established for many tumor types. CD95L expression by malignant cells may counteract the host's antitumor immunity and favors immune escape of the tumor. On this basis, the significance of loss of CD95 and gain of CD95L expression for tumor progression is discussed.

Key words Somatic mutations · Apoptosis · Antitumor immunity · Differentiation · Tolerance · Invasion

Abbreviations *AICD*: Activation-induced cell death · *ALL*: Acute lymphoblastic leukemia · *IFN*: Interferon · *LGL*: Large granular lymphocytic · *MHC*: Major histocompatibility complex · *MMP*: Matrix metalloproteinase · *PCR*: Polymerase chain reaction · *PML*: Promyelocytic leukemia · *RAR*: Retinoic acid receptor · *TCR*: T cell receptor

MARKUS MÜSCHEN
studied medicine in Düsseldorf, Germany, and received research fellowships at the University of Nantes, France, and the Institut Pasteur in Paris, France, in tumor immunology. He joined the group of Klaus Rajewsky at the Institute for Genetics in Cologne, Germany, in 1998 and presently holds a postdoctoral research fellowship of the Cancer Research Institute in New York, USA, with a major research interest in tumor immunology.

ULRICH WARSKULAT
received his Ph.D. from the Department of Physiological Chemistry at the University of Düsseldorf, Germany. He continued his research at the Medical Center of the University of Freiburg, Germany, and is now a member of Dieter Häussingers Department of Gastroenterology, Hepatology and Infectiology at the University of Düsseldorf, Germany. His research interest includes molecular aspects of hepatology and tumor immunology.

MATTHIAS W. BECKMANN
studied medicine and conducted postdoctoral research fellowships in molecular endocrinology, pathology, and oncology in Brussels, Belgium, Freiburg, Germany, Durban, South Africa, Basel, Switzerland, and Chicago, USA. He trained in obstetrics and gynecology in Freiburg, Frankfurt, and Düsseldorf. Presently he is Associate Professor at the Heinrich Heine University, Düsseldorf, with major research areas in molecular medicine in obstetrics and gynecology.

M. Müschen (✉)
Institute for Genetics, Department of Immunology
and Medizinische Klinik I, Universität zu Köln,
LFI E4 R705, Joseph-Stelzmann-Str. 9,
50931 Cologne, Germany
e-mail: markus.mueschen@uni-koeln.de

U. Warskulat
Department of Internal Medicine,
Heinrich-Heine-Universität Düsseldorf, Moorenstr. 5,
40225 Düsseldorf, Germany

M.W. Beckmann
Department of Gynecology and Obstetrics,
Heinrich-Heine-Universität Düsseldorf, Moorenstr. 5,
40225 Düsseldorf, Germany

Introduction

CD95 (Apo-1/Fas) is a cell-surface receptor involved in cell death signaling [1, 2, 3]. The death signal cascade is initiated upon cross-linking of CD95 by its natural ligand (CD95L) [4]. Whereas CD95 expression and susceptibility to CD95L-mediated apoptosis is a common feature of most nonmalignant tissues in the human [5], constitutive expression of CD95L is restricted to a few anatomically well defined structures. Sertoli cells in testis [6], epithelial cells of the anterior chamber of the eye

[7], and Kupffer cells along the hepatic sinusoids [8, 9] constitutively express CD95L. Functional studies have revealed that CD95L expression at these sites confers localized immune privilege [6, 7, 8]. In consequence, these immunoprivileged sites retain a microenvironment of tolerance as infiltrating $CD95^+$ lymphocytes are rapidly killed by apoptosis initiated after CD95 ligation.

The interaction between effector and target cells of CD95L-mediated cytotoxicity can be modulated in several ways. For instance, CD95L can be neutralized by a soluble isoform of the CD95 molecule [10, 11]. Soluble CD95 is generated by alternative splicing and lacks the transmembrane domain which anchors the receptor molecule within the cell membrane of a target cell. Hence, soluble CD95 no longer transduces the death signal after binding to CD95L [10, 11] and competes with transmembrane CD95 for CD95L binding. Competitive CD95L antagonism by soluble CD95 efficiently prevents lymphocyte killing in vitro [8] and *in vivo* [12, 13]. Indeed, aberrant overexpression of soluble CD95 is mechanistically involved in the pathogenesis of certain autoimmune diseases [13, 14, 15], giving way to autoreactive T cells.

In addition to CD95 also CD95L occurs in a soluble form. Soluble CD95L can be generated as a posttranslational modification by proteolytic cleavage by matrix metalloproteinases (MMPs) [16, 17]. Shedding of CD95L by MMPs has been detected in embryonal [18] and squamous cell carcinoma [19], breast cancer cells [20, 21], Kupffer macrophages [8], and sinsusoidal endothelial cells [9], indicating that the occurrence of CD95L in a soluble form is not restricted to malignant cells. Also, after being shed from the cell membrane of effector cells CD95L molecules retain their capacity to induce apoptosis [22, 23]. Compared to membrane bound CD95L, however, the cytotoxic potential of soluble CD95L is significantly diminished [22, 23]. The generation of soluble CD95L molecules raises the possibility of systemic tissue damage which is supported by elevated serum levels of liver enzymes [16] and depletion of peripheral blood T cells [21] in correlation with increased serum levels of soluble CD95L.

There is now broad evidence demonstrating that malignant cells take advantage of aberrant loss of CD95 [5, 18, 19, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37] and gain of CD95L [18, 19, 20, 21, 26, 28, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63] expression compared to their nonmalignant counterparts. Malignant cells in many tumor types aberrantly express CD95L and create an immunoprivileged site, thus escaping the immunosurveillance of the host organism [18, 19, 20, 21, 31, 38, 39, 40, 41, 46, 47, 48, 53, 54, 55, 56, 57, 58, 59]. CD95L expression by cancer cells not only prevents rejection by the immune system but also contributes to tissue damage and metastatic spread [19, 20, 21, 35, 37, 52].

Aberrant CD95 and CD95L expression reflects loss of differentiation in tumor cells

Studies of CD95 and CD95L expression in esophageal and oral squamous cell carcinoma by immunohistochemistry observed a correlation between CD95L expression and loss of nuclear differentiation, suggesting that squamous cell carcinomas acquire high levels of CD95L expression during the dedifferentiation process [33, 59]. A variable fraction of CD95L expressed by malignant cells, however, can be shed from the cell membrane by MMPs [16, 17]. Therefore immunohistochemistry does not necessarily reflect the accurate levels of CD95L expression by malignant cells.

Similar to the findings in squamous cell carcinomas, high levels of CD95L mRNA and protein expression are correlated with dedifferentiation of breast cancer cells [20, 21, 62]. Compared to nonmalignant mammary epithelia, CD95L mRNA levels have been shown to be 310-fold higher in undifferentiated (GIII), 120-fold higher in moderately differentiated (GII), and about 20-fold higher in well-differentiated breast cancer (GI). On the other hand, loss of differentiation of epithelial cells of the mammary gland (fibroadenoma, GI-GIII breast cancer) is correlated with loss of transmembrane CD95 receptor mRNA and protein expression in benign and malignant mammary epithelia. Compared to benign breast tissue, mRNA levels for transmembrane CD95 are diminished in GI breast cancer by 30%, in GII carcinomas by 50%, and in GIII breast cancer by 80% (Fig. 1).

Studying CD95 and CD95L expression in breast cancer and benign mammary tumors at the mRNA level circumvents the detection problem of posttranslational CD95L processing. Functional tests indicate that loss of CD95 expression during dedifferentiation results in loss of sensitivity to apoptosis in dedifferentiated breast cancer cells. On the other hand, increased levels of CD95L expression during the dedifferentiation process are associated with higher cytotoxic activity of breast cancer cells. The tumor cells are able to kill cocultured activated T cells via CD95L, the extent of T cell apoptosis depending on the degree of tissue differentiation [21]. Thus de novo expression of CD95L and loss of CD95 expression are related to loss of differentiation in breast cancer [20, 21, 62].

This correlation has been addressed by an *in vitro* model for differentiation of malignant cells [18]. Human embryonal carcinoma cells are undifferentiated and can progressively acquire a differentiation phenotype of various lineages upon prolonged treatment with all-*trans* retinoic acid. Studies of CD95 and CD95L mRNA expression during all-*trans* retinoic acid induced differentiation of human embryonal carcinoma cells have verified the direct effect of cellular differentiation on both CD95 (positive) and CD95L (negative) expression under experimental conditions [18]. It remains unclear whether loss of differentiation in breast cancer or squamous cell carcinoma cells is due to arrest of the differentiation program (according to the model of all-*trans* retinoic acid induced

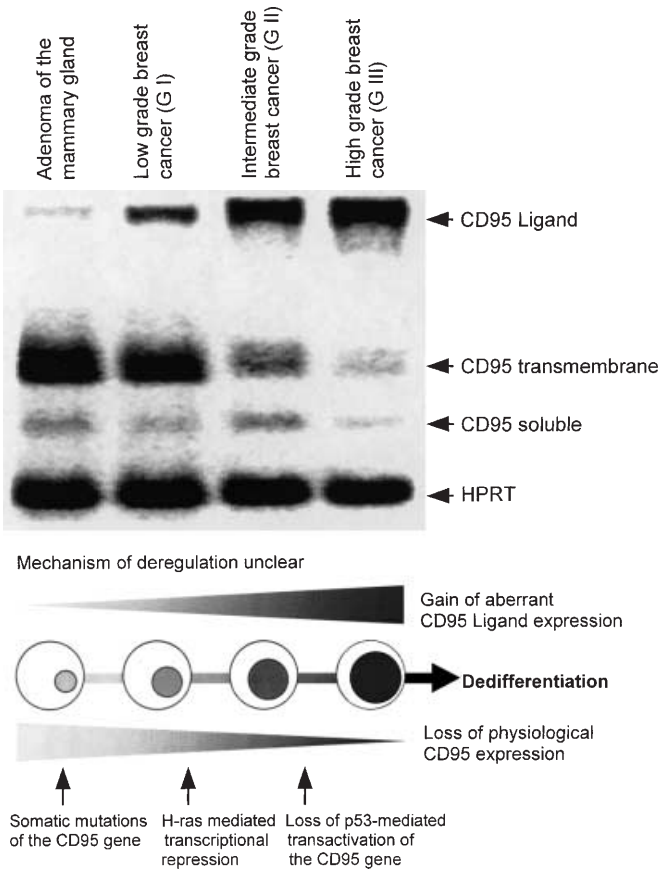

Fig. 1 Deregulation of CD95 and CD95L expression during progression of breast cancer. *Upper panel* Reverse transcriptase PCR products for quantitative amplification of CD95L, transmembrane CD95 isoform, soluble CD95 isoform, and *HPRT* (housekeeping gene) cDNAs are shown. These cDNAs were amplified from mammary fibroadenoma, well differentiated breast cancer (low grade), moderately differentiated breast cancer (intermediate grade) and undifferentiated breast cancer (high grade). *Below* Gain of aberrant CD95L expression and loss of CD95 expression during the dedifferentiation process are depicted. For loss of CD95 expression, possible causes are given

differentiation) or indeed reflects a regression of the malignant cells to a less differentiated stage.

In conclusion, loss of CD95 and gain of CD95L expression vary as a function of the degree of dedifferentiation in malignant cells. The correlation between the degree of malignant progression and levels of CD95L expression suggests that CD95L would be a useful diagnostic and prognostic marker in a number of tumors which are derived from CD95L$^-$ lineages [20, 32, 36, 60]. The specificity of CD95L expression in breast cancer (as opposed to normal breast epithelia), however, was a matter of debate, since one group has reported CD95L expression in both benign and malignant mammary epithelia [64], in contrast to findings by us and others [20, 21, 31, 54, 60]. Since infiltrating T cells are a possible source of CD95L expression, the purity of the analyzed tissue is critical, and appropriate controls are mandatory. This issue has been recognized and appropriately resolved in earlier studies [20, 21, 31, 54], which was not the case in a later study [64] using a "semiquantitative" reverse transcriptase polymerase chain reaction (PCR) approach with amplification conditions under which also few "contaminating" T cells would give rise to a PCR product, most likely explaining the discrepancy between the studies. Thus, we consider CD95L expression as a criterion of malignant transformation in breast cancer as in many other tumor entities (see Table 1).

Mutations of the CD95 gene confer resistance towards CD95L-mediated apoptosis

One of the mechanisms contributing to resistance of malignant cells to CD95L-mediated apoptosis involves mutations of the CD95 gene, which were first encountered in the germline of the so-called *lymphoproliferation* phenotype mice (*lpr*; [65, 66]). These mice develop lymphadenopathy and splenomegaly and are prone to autoimmunity and B cell lymphoma [60, 61, 67]. Further studies have revealed the occurrence of mutations of the CD95 gene in the germline in the human [68, 69, 70, 71, 72, 73] (Fig. 2) as well. In these cases CD95 germline mutations result in autoimmune lymphoproliferative syndrome [68, 69, 70, 71, 72, 73] or Canale-Smith syndrome [74], which are characterized by systemic autoimmunity, generalized lymphoproliferation with dramatic enlargement of liver and spleen, and significantly increased incidence of B cell lymphoma and other malignancies [75, 76].

Somatic CD95 mutations impairing the transduction of the apoptotic signal were first described in lymphoid tumors [77, 78, 79, 80, 81, 82, 83]. In lymphomas derived from antigen-experienced B cells, mutations of the CD95 gene may have been acquired during the germinal center reaction and thus represent traces of somatic hypermutation outside the Ig loci. Somatic hypermutation of non-Ig genes has recently been observed, for example, in the BCL-6 gene [84]. This can be the case in several non-Hodgkin lymphomas originating from mature B cells that carry mutated Ig V region genes. The occurrence of CD95 mutations has been reported in up to 60% of non-Hodgkin lymphomas derived from (post)-germinal center B cells, depending on the entity (Table 2) [77, 78, 79]. In 31 cases of Hodgkin's disease (which derives from a mature B cell in most cases [85, 86]) no allelic loss of the CD95 gene is observed [77]. Since in Hodgkin's disease the tumor clone typically accounts for less than 1% of the tumor mass within a complex admixture of infiltrating T cells, eosinophils, and histiocytes [85], the issue of somatic alterations of the CD95 gene in the Hodgkin and Reed-Sternberg cells is not appropriately addressed analyzing genomic DNA extracted from whole lymph node tissue [77]. Sequence analysis of the CD95 gene from single micromanipulated Hodgkin and Reed-Sternberg cells reveals that somatic mutations impairing CD95 function indeed occur in Hodgkin's disease (M. Müschen, D. Re, A. Bräuninger, M.L. Hans-

Table 1 Role of deranged CD95 and CD95L expression in malignant progression

Characteristic	Tumor	Effect	Reference
Dedifferentiation	Breast cancer	Loss of CD95 expression	5, 20, 21, 24, 62
		Gain of CD95L expression	20, 21
	Burkitt's lymphoma	Deranged CD95 and CD95L expression	30
	Embryonal carcinoma	Loss of CD95 expression	18
		Gain of CD95L expression	18
	Pancreatic carcinoma	Gain of CD95L expression	28
	Squamous cell carcinoma	Gain of CD95L expression	19, 33, 59
Invasion	Breast cancer	Apoptosis of stroma cells	20
	Colon adenocarcinoma	Apoptosis of epithelial cells	37, 41, 47, 52
		Vascular invasion	35
	Hepatocellular carcinoma	Apoptosis of parenchymal cells	38
	Lung cancer	Apoptosis of parenchymal cells	43, 61
	Malignant melanoma	Apoptosis of stroma cells	39, 56, 116
	Renal carcinoma	Vascular invasion	35
	Squamous cell carcinoma	Apoptosis of stroma cells	19
Metastasis	Breast cancer	Correlation of CD95L expression with metastasis	20
	Colon adenocarcinoma	Apoptosis of liver parenchymal cells	37
		Enhanced CD95L expression in metastasis	37, 52
		Vascular invasion	35
	Ewing's sarcoma	Enhanced CD95L expression in metastasis	45, 115
	Malignant melanoma	Correlation of CD95L expression with metastasis	56, 116
Immune escape	Astrocytoma	Killing of tumor infiltrating T cells	42, 64
	Breast cancer	Induction of T cell anergy	21
		Killing of infiltrating T cells	20, 21, 31, 54
	Cholangiocarcinoma	Killing of infiltrating T cells	55
	Embryonal carcinoma	Killing of infiltrating T cells	18
	Esophageal carcinoma	Killing of infiltrating T cells	59
	Gastric carcinoma	Killing of infiltrating T cells	48, 50
	Hepatocellular carcinoma	Killing of infiltrating T cells	38
	Hodgkin's disease	T cell killing	34
	Lung carcinoma	Killing of infiltrating T cells	32, 43
	Malignant melanoma	Killing of infiltrating T cells	39
	Ovarian cancer	Induction of T cell anergy	46
	Pancreatic carcinoma	Killing of infiltrating T cells	28, 57, 58
	Malignant melanoma	Killing of infiltrating T cells	39
	Squamous cell carcinoma	Killing of infiltrating T cells	19
	Thyroid carcinoma	Killing of infiltrating T cells	53
Systemic tissue damage	Breast cancer	Depletion of peripheral blood lymphocytes	21
	Burkitt's lymphoma	Elevated liver enzymes	16
	LGL leukemia	Elevated liver enzymes	16
	Nasal lymphoma	Elevated liver enzymes	106
Resistance to cytostatic drug treatment	Breast cancer	Loss of CD95 function	27
	Burkitt's lymphoma	Loss of CD95 function	30
	Squamous cell carcinoma	Loss of CD95 expression	19
	T-lineage leukemia	Loss of CD95 function	40
Prognosis	Breast cancer	CD95L expression unfavorable	20, 62
	Colon cancer	CD95L expression unfavorable	52
	Esophageal carcinoma	CD95L expression unfavorable	36
	Lung cancer	CD95L expression unfavorable	32
	Malignant melanoma	CD95L expression unfavorable	56

mann, J. Wolf, V. Diehl, K. Rajewsky, and R. Küppers, manuscript submitted).

In B-lineage acute lymphoblastic leukemia (ALL), however, the tumor clone is derived from an immature B cell at an early step of development prior to the onset of somatic hypermutation, which specifically occurs within the germinal center [87]. In 32 cases of B-lineage ALL no mutations of the CD95 gene were encountered [82].

Mutations of the CD95 gene are also found in T-lineage ALL [79, 80, 81, 82] and several tumors of

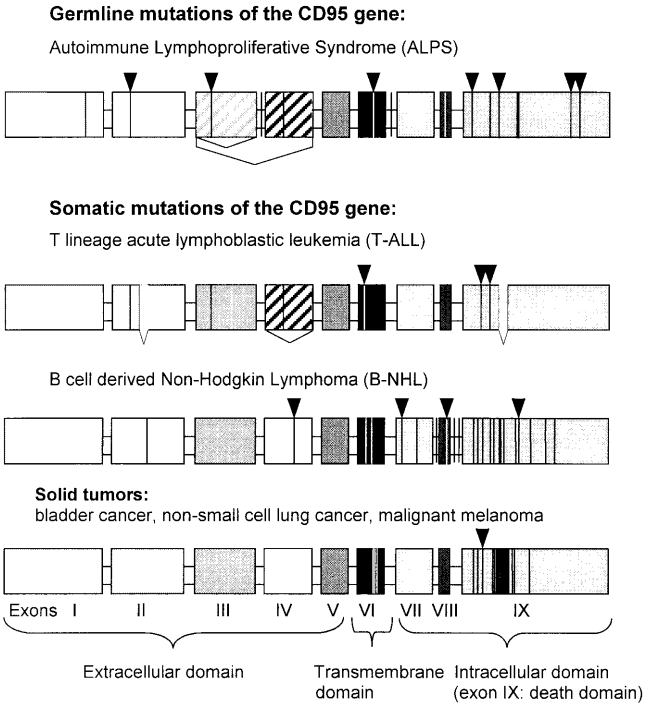

Fig. 2 Germline and somatic mutations of the CD95 gene are related to malignancy. An overview of mutations of the CD95 gene described in the literature [68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 89, 90, 91] and their distribution over the nine coding exons is schematically depicted. Point mutations are depicted by *vertical lines* within the respective exons; *arrowheads* point mutations generating a stop codon or frameshift; *dashed boxes* deletions. In exon 9, the positions of the point mutations are not to scale, as for some nucleotide positions multiple mutations were encountered. *Panel 1* Inherited germline mutations of the CD95 gene leading to autoimmune lymphoproliferative syndrome; *panels 2–4* somatic mutations within T-lineage acute lymphoblastic leukemia cells (*2nd*), B cell derived non-Hodgkin lymphoma cells (*3rd*) and malignant cells of the epithelial lineage (*4th*) are depicted

nonlymphoid lineages, demonstrating that somatic hypermutation is, if at all, not the only mechanism introducing mutations within the CD95 gene. To clarify the contribution of somatic hypermutation to alterations in the CD95 gene it will be interesting to determine whether also normal human B cells can acquire somatic mutations of that gene during the germinal center reaction.

Since T cell receptor V region genes are not subject to somatic hypermutation (at least not in immature T cells giving rise to T-lineage ALL), somatic mutations of the CD95 gene in some cases of T-lineage ALL [81, 82, 83] cannot be explained by a mechanism related to somatic hypermutation. More recently, somatic mutations of the CD95 gene have also been found in solid tumors, namely bladder cancer [89], non-small cell lung cancer [90], malignant melanoma [91], and squamous cell carcinoma [92]. Although in these tumor types, the mutations appear to be clonal in the tumor cells, and some of them involve both alleles, the functional relevance of these findings remains to be established. Apart from mutations by which a translational stop is created, for the majority of the mutations it remains unknown whether they are deleterious (Fig. 2). In solid tumors, loss of heterozygosity has been detected in about 30% of informative cases for at least one polymorphic marker. The detection of biallelic mutations of the CD95 gene in some cases and the frequent occurrence of loss of heterozygosity fit the "two-hit theory" developed by Knudson in 1971 [93], describing the sequential inactivation of both alleles of the retinoblastoma tumor suppressor gene. Notably, loss of heterozygosity is observed in B cell lymphoma and solid tumors only in the presence of deleterious mutations of the CD95 gene targeting the first eight exons. Loss of heterozygosity has so far not been observed in the case of mutations within exon 9 (coding for the death domain), many of which have been shown to exhibit a dominant negative effect.

In the four types of solid tumors the frequency of cases in which the tumor cells harbor a somatically mutated CD95 gene ranges between 4% and 28% (Table 2). Although some mutations might have been missed due to technical matters in the analysis, it is obvious that somatic mutation of the CD95 gene does not constitute a unifying event of malignant transformation in these tumor types. In addition, somatic mutations of the CD95 gene seem to be absent in some other tumor types. In addition to B-lineage acute lymphoblastic leukemia [88], colorectal carcinomas have also been consistently found to lack somatic CD95 mutations or significant allelic loss in two studies [25, 94]. It is an intriguing finding, however, that more than 80% of all mutations of the CD95 gene accumulate in exon 9 which codes for the death domain (Fig. 2). This suggests a hot spot for somatic mutations within exon 9 due to unusual instability of a particular DNA sequence. The assumption of a hot spot is supported by the finding that among 23 individuals which carry four types of solid tumors with a somatically mutated CD95 gene, ten patients harbor tumors which share an identical G→A transition at bp 993 resulting in a replacement of Val→Ile at codon 251 (exon 9; Fig. 2). As the four studies were carried out by the same group [89, 90, 91, 92] the repeated amplification of an identical mutation from ten different patients may be indicative of PCR contamination. In each of the four studies, however, the analysis was thoroughly controlled for PCR contamination by a number of independent repeat experiments and negative controls. The G→A transition at bp 993 was not found in nonmalignant tissue samples from these patients, which argues for the presence of a hot spot for somatic mutation rather than a so far unknown germline polymorphism. An interindividually shared point mutation of the CD95 gene (codon 253; exon 9) was also encountered in two patients with multiple myeloma by another group [78].

Exon 9 encodes the death domain, which is evolutionary highly conserved and has been shown to be necessary and sufficient for the transduction of the apoptotic signal [95, 96]. Given the functional importance of this region, the concentration of CD95 mutations within exon 9 may also indicate that tumor cells are selected for a de-

Table 2 Frequency of somatic CD95 mutations in hematological and solid malignancies

Malignancy	Cases with CD95 gene mutations		Reference
	n	%	
Hematological			
B-lineage ALL	0/32	0	88
T-lineage ALL	2/81	2.5	81
	3/47	6.4	82
	1	n/a	79
	1	n/a	83
Non-Hodgkin lymphomas (overall)	3/70 LOH	4.2	77
	16/150	10.7	80
Anaplastic large-cell lymphoma	1/2	(50.0)a	80
Diffuse large-cell lymphoma	9/43	20.9	80
Follicle center cell lymphoma	2/33	6.1	80
Mucosa-associated lymphoid tissue lymphoma	3/5	(60.0)a	80
	0/18	0	119
Multiple myeloma	7/54	13.0	79
Hodgkin's disease	0/31 LOHb	0	77
	0/2	0	121
	2/14	14.3	Unpublished
Solid tumors			
Bladder cancer	12/43	27.9	89
Colon carcinoma	0/12	0	94
Malignant melanoma	3/44	6.8	91
Non-small-cell lung carcinoma	5/65	7.1	90
Squamous cell carcinoma	3/71	4.2	92

[a] Percentages in parentheses due to small number of cases
[b] LOH was analyzed from whole tissue DNA thus not reflecting the tumor clone (<1% of cells)

fective death domain of CD95 during malignant progression. An argument for selection of these mutations may be derived from the calculation of the ratio for mutations leading to amino acid replacement (R) versus silent (S) mutations (R/S ratio). From a random distribution of mutations one would expect R/S ratios of about 3.0. Therefore it is interesting to note that in non-small-cell lung cancer, bladder cancer, malignant melanoma, and squamous cell carcinoma the overall R/S ratio is 22.0 and thus far from random. Similarly, in 67 somatic mutations of the CD95 gene described in the literature (Fig. 2), there are only three silent mutations, resulting in an overall R/S ratio of 21.3. The effect of mutations within the death domain of the CD95 gene on selection of lymphoma cells has been studied in vitro: truncation of CD95 was shown to confer apoptosis resistance and positive selection to tumor cells harboring at least one copy of the mutant CD95 [96].

More or less severe clinical phenotypes have been observed for some patients with monoallelic CD95 mutations in the germline of exon 9 [71, 72, 73, 76], indicating that monoallelic mutations within the last exon may have detrimental effects despite a functional CD95 gene on the other allele. Monoallelic mutations of the death domain coding exon act in a dominant negative manner, which reinforces their clinical relevance. The dominant negative effect of monoallelic CD95 mutations may be due to the fact that the transduction of the death signal by CD95 requires trimerization of CD95 molecules. Thus, monoallelic expression of a defective CD95 molecule can affect CD95 function as a whole [71]. In line with positive selection of CD95 mutants, loss of heterozygosity has been observed exclusively in the presence of mutations affecting exons 1–8, and not in the case of dominant negative mutations of exon 9 coding for the death domain.

Only somatic point mutations have so far been detected in B cell derived non-Hodgkin lymphomas and solid tumors (Fig. 2). Among germline mutations leading to autoimmune lymphoproliferative syndrome and somatic mutations in T-lineage ALL cells, however, also a number of large deletions and insertions have been identified (Fig. 2). It remains unclear whether this difference reflects distinct mechanisms of mutation within the CD95 gene (e.g., somatic hypermutation versus tumor associated genomic instability) or is simply due to the fact that sequence analysis is based principally on genomic DNA in one case and on transcripts in the other.

Together with the notion of (a) an increased incidence of B cell lymphomas in *lpr/lpr* mice, (b) the observation of an increased risk of lymphoid and solid malignancies in patients with inherited mutations of the CD95 gene, and (c) the finding that tumor cells harboring a truncated CD95 gene are positively selected, these data collectively indicate that CD95 can act as a tumor suppressor gene.

Secondary loss of CD95 function

Since tumor cells in most if not all malignancies lose or reduce their sensitivity to CD95L-mediated apoptosis, other factors must be involved in the impairment of CD95 signaling in addition to deleterious mutations of the CD95 gene (which are observed only in a subset of tumors; Table 2) [25]. For instance, progressive loss of

Table 3 Regulation of CD95 function

Level of regulation	Mechanism	Reference
Transcriptional regulation of CD95	Transactivation of the CD95 promotor by p53	27
	Upregulation of CD95 mRNA expression by IFNg	5, 8, 9, 18, 19
	Inducibility of CD95 transcription by GA binding protein	99
	Inducibility of CD95 transcription by AP-1	99
	Transcriptional downregulation of CD95 mRNA expression by H-ras induced hypermethylation of the CD95 gene	97
Regulation of CD95 signaling	Impairment of CD95 signaling by loss or rearrangement of PML	100
	The bcr-abl translocation confers specific resistance to CD95-mediated apoptosis	101
	The transport of the CD95 protein to the cell membrane is coordinated by P53	98
	CD95 signaling requires functional P53	29

CD95 mRNA and protein expression have been demonstrated during the dedifferentiation process in breast cancer [20] (Fig. 1). However, the somatic mutations of the CD95 gene identified so far do not interfere with transcription. Mutated CD95 mRNA is not counterselected except in the rare cases of stop codons (which result in decreased mRNA stability).

Thus, somatic mutations are very unlikely to fully account for the reduction in CD95 mRNA levels in parallel to malignant progression. Therefore *trans*-acting elements of transcriptional deregulation of CD95 expression have been investigated. Oncogenic H-ras alteration [97] and mutated p53 [27, 29, 98] have so far been identified as being involved in transcriptional downregulation of CD95 in malignant cells, whereas CD95 transcription has been demonstrated to be upregulated by GA-binding protein and AP-1 [99]. Oncogenic H-ras entirely suppresses CD95 mRNA expression through hypermethylation of the CD95 gene [97] and confers resistance to CD95L-mediated apoptosis which can be reverted upon inhibition of DNA methylation. A strong correlation between CD95 expression and p53 wt function has been observed in a number of cell lines of human hepatocellular and gastric carcinoma and colon and breast cancer [27, 29] (Table 3). Investigating p53-mediated transactivation of CD95, the group of Peter H. Krammer identified one p53-responsive element within the first intron of the CD95 gene and three putative elements within the CD95 promotor [27]. Functional assays have revealed that the intronic element confers transcriptional activation of the CD95 gene by p53 and cooperates with the responsive elements in the promotor. In contrast to mutated p53, wt p53 binds and transactivates the CD95 gene.

Since accumulation of mutations within the p53 gene and deregulation of H-ras signaling are frequent events during malignant progression, it is conceivable that these events together progressively silence CD95 expression (Fig. 1). In addition to transcriptional regulation of CD95 expression by p53, the function of the CD95 protein is further regulated by p53, since the transport of CD95 protein to the cell surface is also coordinated by p53 [98] (Table 3). Finally, some oncogenes have been shown to interfere with the transduction of the apoptotic signal downstream of CD95 ligation [100, 101]. Loss of the promyelocytic leukemia associate gene (PML) or its rearrangement to retinoic acid receptor (RAR) α (resulting in the PML-RARα fusion molecule which is typically expressed by PMLs) impaired CD95L-mediated apoptosis most likely by inhibition of caspase 3 [100]. PMLs harboring the PML-RARα translocation are thus resistant to CD95L-mediated apoptosis, lacking function of CD95 [100]. Loss of sensitivity to CD95L-mediated apoptosis has been observed in chronic myelogenous leukemia cells which express the Bcr-Abl fusion molecule (involving the oncogenic Abl kinase) [101]. The transduction of CD95 signaling can be fully restored by specific inhibition of the Abl kinase, demonstrating the oncogenic antiapoptotic effect of abl downstream of CD95 ligation in the leukemia cells [101] (Table 3).

The implication of CD95 in Ras- and p53-dependent signaling pathways is reminiscent of the genetic model for tumorigenesis of colorectal cancer developed by Fearon and Vogelstein [102], describing a sequence of deleterious mutation events involving the APC, K-Ras, DCC, and p53 genes.

CD95L mediates immune escape of malignant cells

CD95L expression under physiological conditions (e.g., in cytotoxic T cells) is frequently self-limiting as CD95L-expressing cells are usually themselves targets of CD95L-mediated cytotoxicity. Cytotoxic T cells increase expression of both CD95 and CD95L upon activation and subsequently undergo “suicide” or “fratricide” by CD95 ligation. This phenomenon, called activation-induced cell death (AICD), is critical to retain the balance of pro- and anti-apoptotic agents in terms of cellular homeostasis [103]. However, malignant cells escape “suicidal” AICD as they lose or experience largely reduced CD95 expression. Thus loss of CD95 expression in malignant cells is crucial not only to protect them from rejection by cytotoxic immune cells but also to enable them to exhibit CD95L-mediated cytotoxicity without induction of “suicide” or “fratricide.” Therefore, it is

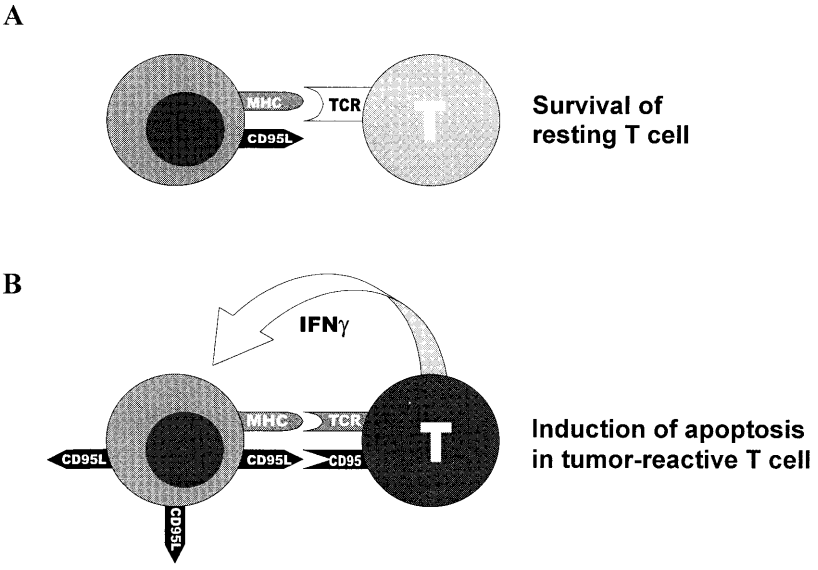

Fig. 3 Model for selective killing of tumor-antigen specific T cells by CD95L-expressing tumor cells. Resting T cells in the proximity of tumor cells are spared from CD95L-mediated cytotoxicity. These T cells do not recognize (tumor-) specific antigen, are not activated, and express CD95, if at all, at low levels, resulting in low sensitivity towards CD95L-mediated cytotoxicity (**A**). Tumor-antigen specific T cells are activated upon MHC-TCR interaction, express CD95 at high levels, and release high levels of IFNγ which increases expression of CD95L by the tumor cells. Due to the close MHC-TCR interaction, these activated T cells are also targeted by CD95L -mediated cytotoxicity and subsequently undergo apoptosis (**B**)

remarkable that many tumor types have an inverted the pattern of CD95 and CD95L expression to that is usually seen in their nonmalignant counterparts ($CD95^+$, $CD95L^-$). As summarized in Table 1, aberrant expression of CD95L is a common feature of malignant cells, which can result in tissue invasion, metastatic spread, and immune escape.

In the latter case, CD95L-expressing tumor cells are thought to kill activated T cells by CD95 ligation. Indeed, in a number of tumor types tumor-infiltrating T cells exhibiting activated phenotype [104] have been found to undergo apoptosis in situ in the area of $CD95L^+$ tumor cells [20, 21, 38, 39, 46, 47, 48, 53, 59] (Figs. 3, 4A–D). Killing of other immune effector cells (e.g., B cells and macrophages) by CD95L-expressing tumor cells has not yet been reported. Furthermore, abrogation of alloantibody production against CD95L-expressing allografted tumor cells by CD95L-mediated cytotoxicity is caused indirectly by killing of $CD4^+$ T cells rather than B cells [105]. The failure of tumor-specific B cell response is most likely due to the lack of efficient T cell help, since activated tumor reactive $CD4^+$ T helper cells are killed upon encounter with the tumor cells. However, among the T cells killed by CD95L-expressing breast cancer cells there is no apparent preference for either the $CD4^+$ (helper) or the $CD8^+$ (cytotoxic) subset [21]. Interestingly, CD95L mRNA expression in breast cancer is closely correlated with depletion of both $CD4^+$ and $CD8^+$ peripheral blood T cells in the breast cancer bearing patients [21]. This may suggest a relationship between CD95L expression by breast cancer and systemic immunosuppression. Although in addition to CD95L other agents associated with the severity of the tumor disease unquestionably contribute to the reduction in $CD4^+$ and $CD8^+$ lymphocytes, it appears conceivable that peripheral blood lymphocytes are prone to damage by soluble CD95L that is shed from tumor cells, as described in other malignancies.

CD95L-mediated T cell killing by tumor cells seems to be selective for activated rather than resting T cells (Fig. 3). Activated T cells are more sensitive towards CD95L-mediated apoptosis than resting ones (frequently resulting in AICD). T cells are activated upon engagement of their T cell receptor (TCR) by a specifically recognized (tumor cell) antigen within a major histocompatibility complex (MHC). Upon recognition of a defined tumor cell antigen by the TCR-MHC interaction, the T cell becomes activated and thus prone to CD95L-related cytotoxicity. This fatal interaction is further enhanced by interferon (IFN)γ, which is specifically secreted by activated T cells [21] (Fig. 3). IFNγ increases CD95L expression in several breast cancer and squamous carcinoma cell lines, which results in substantially higher levels of CD95L-mediated apoptosis in activated T cells [21]. However, resting T cells are not susceptible to CD95L-mediated cytotoxicity of breast cancer cells. Thus upregulation of CD95L in tumor cells in response to IFNγ may counterselect activated tumor infiltrating T cells and favor T cell anergy (Fig. 3).

$CD95^+$ activated T cells are killed either by direct contact with breast cancer cells or incubated in culture supernatants. This indicates that CD95L expressed by breast cancer cells is an active inducer of apoptosis in its membrane bound and, to lesser extent, in its soluble form. Soluble CD95L is generated as a posttranslational modification by MMP-mediated cleavage from the cell membrane [16, 17, 21, 38]. Thus the role of MMPs in CD95L processing is established in a number of tumor types.

Inhibition of CD95L shedding by MMP inhibitors has been shown to cause a marked accumulation of CD95L protein in human Tera-2 embryonal carcinoma cells [18] and MCF-7 breast cancer cells [21]. This accumulation was paralleled by an increase in CD95-specific apoptosis in activated T cells that were cocultured in the presence of $CD95L^+$ Tera-2 or MCF-7 cells. However, superna-

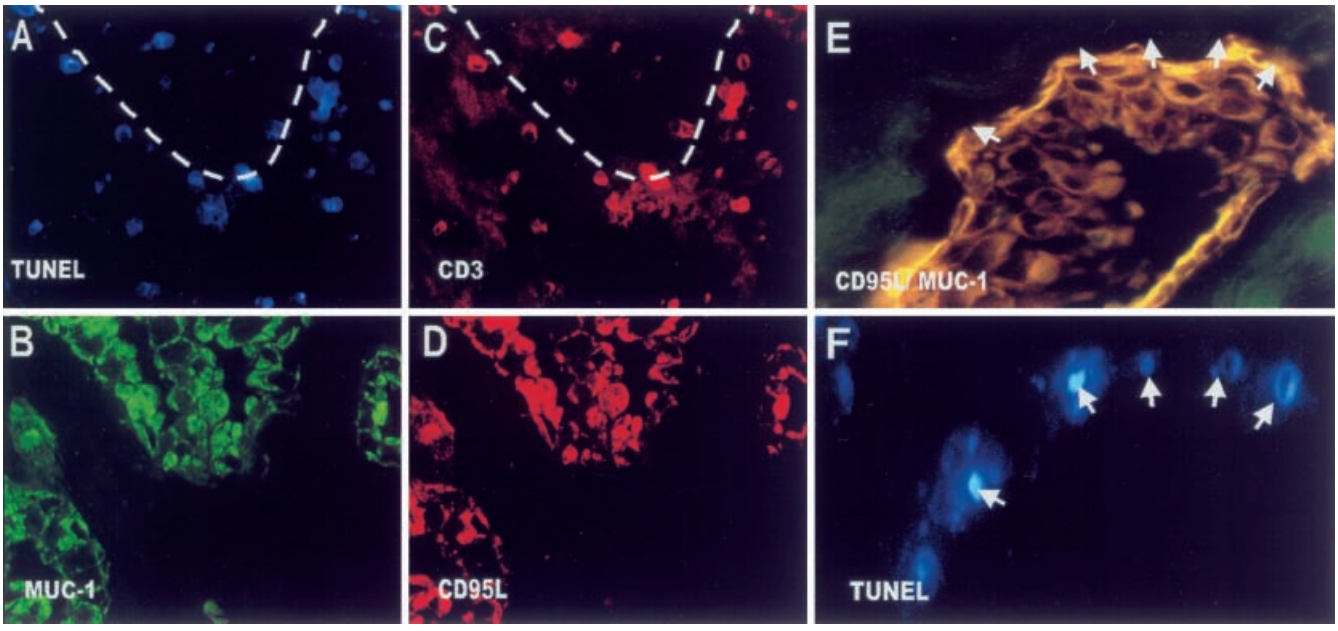

Fig. 4 Cytotoxic effect of CD95L expression in breast cancer on the peritumoral microenvironment. Tissue sections of invasive breast cancer were stained for DNA fragmentation [TdT-mediated dUTP nick end labeling (TUNEL), *blue staining*; **A**, mucin-1 (CA 15-3, *green*; **B**), CD3 (common T cell antigen, *red*; **C**), and CD95L (*red*; **D**) expression using immunofluorescent-labeled antibodies. CD95L expression is specific for and restricted to the malignant cells. The vast majority of the tumor-infiltrating T cells ($CD3^+$; **C**) have already undergone apoptosis ($TUNEL^+$; **A**). Another breast cancer section was double-stained for mucin-1 (CA 15-3, *green*; **E**) and CD95L (*red*; **E**). The staining was visualized by confocal laser microscopy. Superposition of *red* (CD95L) and *green* (mucin-1) fluorescent signals results in *yellow* fluorescence (**E**). CD95L expression is enhanced at the border between the invasive tumor spike and the surrounding stroma tissue and is congruent with the arrangement of apoptotic ($TUNEL^+$; **F**) peritumoral stroma cells (**E**, **F**; *arrowheads*)

tants derived from malignant cells did not induce CD95L-mediated apoptosis in activated T cells when the tumor cells were pretreated with a MMP inhibitor [18, 21].

The fact that CD95L circulates in the periphery after MMP-mediated cleavage implies systemic tissue damage and immunosuppression. Functional studies have shown that the cytotoxicity of soluble CD95L is less than that of membrane-bound CD95L but was still sufficient to induce significant apoptosis in $CD95^+$ bystander cells [22, 23]. Indeed, systemic effects of localized tumors are a frequent feature of the clinical course and are significantly correlated with serum levels of soluble CD95L [16]. However, it remains to be established whether the association between serum levels of liver enzymes, cachexia, fever, and peripheral blood lymphocyte depletion and the respective serum levels of soluble CD95L is merely coincidental rather than of mechanistic importance.

Sera from healthy donors do not contain detectable levels of soluble CD95L, whereas those from patients with large granular lymphocytic (LGL) leukemia and Burkitt's lymphoma do [16]. These malignant cells constitutively express CD95L, whereas peripheral natural killer cells from healthy donors express CD95L only on activation. This suggests that the systemic tissue damage seen in most patients with LGL leukemia and Burkitt's lymphoma is due to soluble CD95L [16]. In a clinical case of an aggressive nasal lymphoma high serum levels of soluble CD95L were correlated with marked liver damage and pancytopenia [106]. CD95L expression by lung carcinomas may also have effects on the immune system beyond the tumor site [43].

CD95L mediates tolerance of tumor cells but rejection of allografts

Under physiological conditions CD95L can mediate localized tolerance and is involved in the creation and maintenance of immunoprivileged sites [107]. This is the case, for example, in the anterior chamber of the eye, in the testis, and along the hepatic sinusoids where epithelial cells, Sertoli cells, and Kupffer cells [6, 7, 8, 9] have been identified as the source of local CD95L expression, respectively.

Malignant cells aberrantly expressing CD95L also create an immunoprivileged site thus preventing the host's immune system from tumor rejection. Accordingly, a role for CD95L in allograft tolerance has been established [6, 108]. In contrast to these findings, CD95L expression in allografts has been found to precipitate acute rejection within 7 days after allotransplantation [109, 110]. Also, CD95L expression in tumor cells is reported to induce rejection and not tolerance of allografted tumors under certain conditions [111]. The ambiguity of CD95L expression has further been exemplified in two $CD95L^+$ subclones derived from a colon carcinoma [49]. Whereas one of these $CD95L^+$ subclones estab-

lished active tolerance and formed an invasive colon carcinoma, the second subclone was rejected despite its functional CD95L expression [49]. These observations led to the hypothesis that the mere expression of CD95L in the presence of antigenic stimuli does not readily determine the type of immune response (i.e., tolerance vs. rejection). Therefore the role of accessory immunomodulating molecules was investigated. Comparing in vivo models for rejection and tolerance in the context of high levels of CD95L expression, transforming growth factor β was identified as a critical cofactor for the establishment of (tumor) tolerance [63, 112, 113]. On the other hand, expression of IFNγ in addition to CD95L has been shown to switch to acute rejection by activating neutrophils and macrophages [114]. In conclusion, CD95L-expressing tumors do not necessarily evade immunosurveillance unless they coexpress an appropriate tolerance signal (e.g., transforming growth factor β).

CD95L favors tissue invasion and metastatic spread

In various tumors accumulation of the CD95L protein is markedly enhanced in invasive tumor spikes especially in the area of the border between invasive tumor tissue and the surrounding stroma cells (Fig. 4E) and colocalized with the arrangement of apoptotic stroma or tumor infiltrating T cells in immediate vicinity of the tumor [19, 20, 38] (Fig. 4F). From these observations one might hypothesize that in the zone of tumor invasion adjacent stroma-, parenchymal- or tumor-infiltrating T cells are targeted by tumor cells bearing CD95L on their cell membrane. CD95L-expressing tumor cells may upregulate CD95L mRNA levels by direct contact with CD95-bearing cells from the antigenic host organism, thus taking advantage of CD95L expression as a factor of tissue invasion.

CD95L expression could conceivably facilitate the establishment of primary tumors or metastases at sites at which the indigenous cells express CD95 receptor and can therefore be rendered subject to CD95L-mediated cytotoxicity [41]. Indeed, metastatic colorectal tumor cells express CD95L more frequently and abundantly than the primary carcinomas [52]. In addition, the CD95 pathway is involved in the promotion of local growth of hepatic colorectal cancer metastases by inducing apoptotic cell death in normal hepatocytes at the tumor margin [32]. This mechanism has been further corroborated by in vitro studies demonstrating that metastatic colon carcinoma cells are able to induce CD95L-mediated apoptosis of primary human hepatocytes in coculture cytotoxic assays. CD95L expression at the margin of colorectal liver metastases induces apoptosis in surrounding $CD95^+$ hepatocytes, facilitating the invasion of the tumor into the surrounding liver parenchyma [32].

Further, metastatic Ewing's sarcomas express both membrane-bound and soluble CD95L at substantially higher levels than the primary tumors, suggesting that CD95L contributes to metastasis of Ewing's sarcoma in its membrane-bound and soluble forms [115]. An association of increased levels of CD95L expression with metastatic phenotype has also been found in malignant melanoma [116]. Loss of CD95 function in malignant melanoma cells has been shown to be causally linked to metastatic progression [117]. Injection of mouse melanoma cells expressing CD95L in wild-type mice leads to rapid tumor formation, whereas tumorigenesis is delayed in CD95-deficient *lpr* mutant mice, in which bystander cells of the host cannot be killed by CD95 ligation [117]. The advantage of tumor cells over the host arising from secondary CD95 resistance during malignant progression is equalized when tumor cells are injected in a *lpr* (i.e., germline CD95-deficient) background. Thus, metastatic progression is determined by the relative difference of tumor cells and the host in terms of apoptosis sensitivity in the CD95 system rather than by the absolute levels of CD95 and CD95L expression found in the tumor cells.

Conclusions

Loss of CD95 and gain of aberrant CD95L expression is a common feature of malignant transformation. Loss of CD95 expression is thereby a prerequisite of aberrant CD95L expression, which would otherwise induce "suicide" among tumor cells. The notion of loss of CD95 function as an oncogenic event is further supported by the observation that other factors implicated in malignant progression (i.e., mutation of the p53 and H-ras genes) [102] downregulate CD95 expression. Furthermore, germline mutations of the CD95 gene in the human are associated not only with lymphoproliferation and autoimmunity but also with a high risk of lymphoid and solid tumors. In a fraction of both lymphoid and solid tumors clonal somatic mutations of the CD95 gene are also found. These data collectively indicate that CD95 can act as a tumor suppressor gene.

Comparing CD95 to various "classical" tumor suppressor genes, however, demonstrates a number of differences. CD95 neither interferes with the regulation of the cell cycle and cellular proliferation, such as the p53 or retinoblastoma (*RB*) genes [118], nor acts as a transcription factor, as opposed to RAS proteins and *BRCA1* [118]. Finally, CD95 is not implicated in DNA repair as MSH2 or the *Xeroderma pigmentosum* (*XP*) genes [118].

In contrast to CD95, "classical" tumor suppressor genes do not require interaction with a physiological ligand. Thereby, deregulation of the CD95/CD95L system in malignant cells (Table 1) involves not only loss of CD95 expression but also aberrant overexpression of CD95L. In addition to CD95, occupancy by a specific ligand is also important for activation of signaling pathways of a number of hormone receptors, for example, the RAR family. However, in the cases of RARs, ligand-receptor interactions do not determine a role as effector or target cell in terms of apoptosis and cytotoxicity. Although also some "classical" tumor suppressor genes (e.g., *p53*) confer susceptibility to apoptosis, the

commitment to a role as a cytotoxic effector or target cell by either CD95L or CD95 expression appears to be a unique feature of this ligand-receptor pair.

Acknowledgements We are indebted to Prof. Dr. Hans Georg Bender (Universitätsfrauenklinik, Heinrich-Heine-Universität, Düsseldorf, Germany), Dr. Maria-Cristina Cuturi, Dr. Régis Josien, and Prof. Dr. Jean-Paul Soulillou (INSERM Unité 437, Nantes, France), Dr. Jos Even (Département d'Immunologie; Institut Pasteur Paris, France), Prof. Dr. Dieter Häussinger (Klinik für Gastroenterologie, Hepatologie und Infektiologie, Heinrich-Heine-Universität, Düsseldorf, Germany), and Prof. Dr. Dr. hc Helmut Sies (Institut für Physiologische Chemie I, Heinrich-Heine-Universität, Düsseldorf) for support and critical discussion. Markus Müschen is supported by a postdoctoral fellowship from the Cancer Research Institute (New York, USA; Tumor Immunology Program).

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