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Inverse Relation of Fas-Ligand and Tumor-Infiltrating Lymphocytes in Angiosarcoma

Indications of Apoptotic Tumor Counterattack

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Fas and Fas-L regulate immune responses through the induction of cell death. Fas-L is commonly expressed in activated immune cells and in the endothelium. In the latter it contributes to the inhibition of transvascular cell migration by the induction of apoptosis in Fas-bearing lymphocytes. Here we investigated whether the Fas/Fas-L system may regulate lymphocyte invasion into angiosarcomas. Fas and Fas-L expression was quantitatively determined in different grade angiosarcomas ($n = 40$) and related to the number of extravasated tumor-infiltrating lymphocytes (TILs). Fas expression was detected in <50% of the cases. In positive tumors both the number of Fas-positive cells and the staining intensity were highly variable and did not correlate with the number of TILs, the mean time of survival, and the histopathological tumor grade. By contrast, Fas-L expression was detected in >70% of the cases and the relative numbers of Fas-L-positive cells correlated inversely with the numbers of CD3- and CD8-positive TILs ($P \leq 0.004$). The survival times of patients with high Fas-L-expressing angiosarcomas were significantly reduced as compared to patients with low Fas-L-expressing tumors. Our results show that angiosarcomas with low Fas-L expression are characterized by numerous TILs, whereas sarcomas with high Fas-L expression show significantly reduced numbers of TILs. These results suggest that the Fas/Fas-L system may repress TIL invasion into angiosarcoma and by this may contribute to the evasion of the anti-tumor immune surveillance of angiosarcoma in the course of an apoptotic tumor counterattack mechanism. *(Am J Pathol 2001, 159:963–970)*

Fas (CD95/APO-1) is a cell surface receptor of the nerve growth factor/tumor necrosis factor receptor superfamily. Its ligand, Fas-L, is structurally related to tumor necrosis factor, lymphotoxin, and CD40 ligand. Fas-L is membrane associated and can be released from the cells in a soluble form by proteolysis. Binding of Fas-L to Fas leads to trimerization of the receptor and to the transmission of apoptotic signals via the so-called "death domain," resident in the cytoplasmic domain of Fas. Fas and Fas-L play a key role in the regulation of apoptosis within the immune system, especially in lymphocyte development, anti-viral immune responses, and the elimination of tumor cells. Malfunction of the Fas/Fas-L pathway can cause lymphoproliferative disorders and acceleration of autoimmune diseases.[1,2]

Fas and its ligand are highly expressed in activated T-cells, natural killer cells, CD34-positive stem cells, and several other nonimmune cells including endothelial cells (ECs).[1–9] Fas-L expression in ECs has been detected in primary cultures of ECs and in tissues in the endothelium of arteries in the rabbit ear and in immunoprivileged sites, such as the human eye and testis.[10–13] It has been shown that endothelial Fas-L induces apoptosis in Fas-expressing immune cells adhering to the vessel wall. This mechanism may contribute to the control of leukocyte extravasation into tissues.[10–13]

ECs in addition to Fas-L also express Fas, both in culture and in tissues in the blood vessels of the central nervous system, the placenta, and the skeletal muscle.[14] However, co-expression of Fas and Fas-L does not induce apoptosis in ECs.[10,11,15] These results suggest that EC-derived tumors may be resistant to cell-mediated anti-tumor defense mechanisms targeting the Fas/Fas-L system. The expression of Fas/Fas-L in EC-derived tumors has so far not been investigated.

Here we investigated the expression of Fas and Fas-L in 40 angiosarcomas with different histopathological tumor grades. Fas protein was detected in <50% of the

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angiosarcomas. In the positive cases it was expressed in variable numbers of tumor cells and did not correlate with any parameter investigated in this study. By contrast, the level of Fas-L expression correlated significantly with a reduction of the numbers of CD3-positive and CD8-positive tumor-infiltrating lymphocytes (TILs). These findings suggest that the angiosarcoma cells may evade cellular anti-tumor responses of the host by a Fas-L-mediated apoptotic counterattack on TILs.

Materials and Methods

Patients

The angiosarcoma collective ($n = 40$) was composed of 18 females and 22 males (median age, 55 years; age range, 24 to 87 years). The localization of angiosarcomas included the extremities ($n = 11$, 27.5%), breast ($n = 6$, 15%), head ($n = 4$, 10%), heart, thyroid and liver (each $n = 3$, each 7.5%), sternum and retroperitoneum (both $n = 2$, both 5%) as well as the diaphragm, trunk, parotid gland, lung, pancreas, and pelvis (each $n = 1$, each 2.5%). The known facultative predisposing factors for the development of angiosarcoma in the cases studied were: chronic lymphedema in three cases and postirradiation status in two cases. Thyroid angiosarcomas are known to show a predilection for inhabitants of mountainous regions (such as the Bavarian alpine region) with iodine deficiency and development of long-standing nodular goiter.[16] Anamnestical data on an occupational exposure to thorotrast (thorium dioxide), arsenic solutions, or vinyl chloride, which may be associated with the development of angiosarcomas were not found. Median survival was 339 days after diagnosis and survival ranged from 18 to 5446 days.

Histopathology

Surgical specimens were collected at the Institute of Pathology (Ludwig Maximilians University, Munich, Germany) in the period between 1983 and 2000. The material had been fixed in buffered formalin (pH 7.0) for 24 to 48 hours and embedded into a low melting paraffin (Paraplast; Vogel, Giessen, Germany). The basic morphological diagnosis and classification of the cases were performed according to the criteria of Enzinger and Weiss,[17] and as described earlier.[18] All angiosarcomas were graded according to the criteria of Coindre and colleagues.[19] Grading resulted in 6 low-grade angiosarcomas (15%), 15 intermediate-grade sarcomas (37.5%), and 19 high-grade sarcomas (47.5%) (Table 1).

Immunohistochemistry

Consecutive 3-μm sections were cut and mounted on sialinized slides (Superfrost Plus; Menzel-Gläser, Braunschweig, Germany). Sections were dewaxed in xylene and rehydrated. Sections for CD95-DX2 and CD95-Apo-1 immunohistochemistry were immersed in Target retrieval solution 6 (Dakopatts; DAKO, Glostrup, Denmark) and subjected to microwave treatment (800 W, 3 × 10 minutes) for antigen retrieval. Microwave treatment of sections for Fas-L (clone 33), Fas-L (clone G247), and CD8 immunohistochemistry was performed in Glyka (Innovative Diagnostik Systeme, Hamburg, Germany), Protex I (Quartett, Berlin, Germany), Target unmasking fluid (TUF, DAKO), respectively. Sections for CD3 staining were treated 30 minutes at room temperature with 0.1% protease Type XXIV (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany).

Avidin-Biotin Technique

The following antibodies were used with the avidin-biotin technique: the monoclonal mouse anti-human CD95/Fas antibody, clone DX2 (DAKO) reacts with the 48-kd APO-I/Fas protein in Western blot analysis. This antibody was used in a final working concentration of 4 μg/ml (incubation time, 60 minutes). The monoclonal mouse anti-human CD95/Fas antibody, clone APO-1(DAKO) reacts specifically in flow cytometric assays with human Fas-transfected cell lines but not with untransfected parental cell lines. This antibody was used in a final working concentration of 82 μg/ml (incubation time, 60 minutes). The monoclonal mouse anti-human Fas-L antibody, clone 33 (BD Transduction Laboratories, Lexington, KY) was raised to residues 116 to 277 of human Fas-L. This antibody was used in a final working concentration of 0.25 μg/ml (incubation time, 60 minutes). The monoclonal mouse anti-human Fas-L antibody, clone G247 (BD Pharmingen, San Diego, CA) was raised to a recombinant protein containing the external domain of human Fas-L. This antibody was used in a final working concentration of 0.6 μg/ml (incubation time, 60 minutes).

Detection of bound primary antibodies was done by standard immunohistochemical streptavidin-biotin-peroxidase technique[20] using commercially available staining kits. For CD95/Fas, clone APO-1 and clone DX2, the Universal DAKO LSAB kit and for Fas-L (clone 33, clone 247) the Vectastain Elite ABC kit (Vector Laboratories, Inc., Burlingame, CA) were used.

Alkaline Phosphatase Anti-Alkaline Phosphatase (APAAP) Technique

The following antibodies were used with the APAAP technique: the polyclonal rabbit anti-human CD3 antibody (DAKO) reacts with the intracytoplasmic portion of the CD3 antigen expressed by T cells. This antibody was used in a final working concentration of 2 μg/ml (incubation time, 30 minutes). The monoclonal mouse anti-human CD8 antibody (DAKO) reacts with a 32-kd protein equivalent to the CD8 molecule as indicated by immunoprecipitation. This antibody was used in a final working concentration of 3 μg/ml (incubation time, 60 minutes).

Detection of bound primary antibodies was done with a standard APAAP method[21,22] using a commercially available APAAP system (DAKO).

Table 1. Immunohistochemical Results and Clinical Parameters

No.	TILs (HPF)		Fas-L [PPC (SI)]		Fas [PPC (SI)]		Survival month	Tumor grade	Age	Localization
	CD8	CD3	C: G 247	C: 33	C: Apo-1	C: Dx-2				
Low TILs										
1	0–1	0–1	90 (H)	80 (H)	0 (−)	0 (−)	1	3	57	Liver
2	0–1	0–1	80 (M)	80 (M)	0 (−)	0 (−)	29	3	59	Thyroid
3	0–1	0–1	80 (H)	80 (L)	0 (−)	0 (−)	2	3	73	Extremities
4	0–1	0–1	80 (H)	80 (H)	0 (−)	10 (L)	1	3	70	Extremities
5	0–1	2–5	80 (H)	70 (M)	0 (−)	0 (−)	16	3	29	Breast
6	0–1	0–1	80 (H)	60 (M)	60 (M)	40 (M)	83	2	42	Thyroid
7	0–1	2–5	70 (M)	60 (M)	0 (−)	10 (L)	7	3	44	Heart
8	0–1	6–10	70 (M)	10 (L)	0 (−)	0 (−)	18	1	59	Retroperitoneum
9	0–1	2–5	60 (M)	70 (H)	0 (−)	0 (−)	8	3	36	Extremities
10	0–1	0–1	60 (H)	70 (H)	60 (L)	50 (L)	4	3	59	Extremities
11	0–1	6–10	50 (M)	30 (L)	20 (L)	40 (L)	42	1	27	Sternum
12	0–1	>10	40 (M)	40 (L)	40 (M)	40 (H)	47	2	29	Sternum
13	0–1	2–5	40 (L)	70 (M)	0 (−)	0 (−)	125	2	45	Pancreas
14	0–1	2–5	40 (L)	70 (M)	0 (−)	0 (−)	6	3	49	Heart
15	0–1	2–5	40 (L)	70 (H)	20 (L)	0 (−)	5	3	44	Heart
16	0–1	2–5	40 (L)	60 (M)	0 (−)	0 (−)	18	2	34	Diaphragm
17	0–1	2–5	40 (L)	60 (L)	60 (L)	60 (L)	2	2	36	Liver
18	0–1	2–5	30 (L)	10 (L)	10 (L)	10 (L)	3	3	24	Breast
19	0–1	2–5	20 (L)	40 (H)	0 (−)	10 (L)	6	3	41	Extremities
20	0–1	2–5	0 (−)	80 (M)	20 (H)	0 (−)	7	3	37	Extremities
21	0–1	0–1	0 (−)	50 (M)	0 (−)	0 (−)	6	2	60	Liver
22	0–1	2–5	0 (−)	0 (−)	0 (−)	0 (−)	16	3	87	Breast
23	2–5	2–5	70 (M)	80 (H)	10 (L)	0 (−)	1	2	75	Extremities
24	2–5	0–1	60 (M)	20 (L)	0 (−)	0 (−)	13	3	75	Extremities
Median	0–1	2–5	55 (M)	65 (M)	0 (−)	0 (−)	7	3	45	–
High TILs										
25	6–10	2–5	40 (M)	20 (L)	30 (L)	10 (L)	3	2	35	Trunk
26	6–10	2–5	20 (M)	40 (L)	0 (−)	0 (−)	8	3	59	Thyroid
27	6–10	6–10	10 (L)	0 (−)	40 (L)	0 (−)	179	1	53	Pelvis
28	6–10	>10	0 (−)	10 (L)	10 (L)	0 (−)	32	2	59	Breast
29	>10	>10	70 (L)	0 (−)	0 (−)	0 (−)	10	2	68	Extremities
30	>10	>10	60 (H)	0 (−)	0 (−)	0 (−)	111	2	32	Lung
31	>10	6–10	50 (M)	40 (L)	30 (M)	0 (−)	36	2	57	Parotis
32	>10	>10	40 (M)	30 (L)	10 (M)	10 (L)	38	1	66	Head
33	>10	6–10	30 (L)	20 (L)	20 (M)	80 (M)	12	2	70	Extremities
34	>10	>10	0 (−)	30 (L)	0 (−)	0 (−)	39	1	73	Head
35	>10	>10	0 (−)	20 (M)	0 (−)	0 (−)	9	3	57	Head
36	>10	>10	0 (−)	20 (L)	40 (L)	30 (L)	26	3	53	Breast
37	>10	>10	0 (−)	20 (L)	0 (−)	0 (−)	7	3	32	Extremities
38	>10	>10	0 (−)	10 (M)	0 (−)	0 (−)	8	1	79	Head
39	>10	>10	0 (−)	0 (−)	30 (L)	0 (−)	51	2	32	Breast
40	>10	>10	0 (−)	0 (−)	0 (−)	0 (−)	12	2	57	Retroperitoneum
Median	>10	>10	5 (−/L)	20 (L)	5 (−)	0 (−)	19	2	57	–

Immunohistochemical staining results of angiosarcomas (n = 40) with antibodies to CD8, CD3, Fas-L (clone 33; clone G247), and CD95-Fas (clone APO-1; clone DX2) as well as clinical data on the time of survival (in months), the histopathological tumor grade, the age of patient, and the localization of the sarcoma. For Fas-L and Fas immunohistochemistry the percentage of positive tumor cells (PPC: 0% negative, 10% of the cells positive, 20% of the cells positive, up to 80% of the cells positive) and the staining intensity (SI, negative; L, low; M, intermediate; H, high) are shown. The numbers of tumor-infiltrating CD8- or CD3-positive lymphocytes (TILs) per HPF have been classified (0 to 1 TILs/HPF, 2 to 5 TILs/HPF, 6 to 10 TILs/HPF, ≥10 TILs/HPF). The median values of the different results obtained for low and high numbers of TILs (0 to 5 *versus* 6 to ≥10) have been calculated and are shown.

Controls

To prove staining specificity the following controls were included in all experiments: for a negative control the primary antibody was replaced with mouse isotype immunoglobulins. No immunohistochemical staining was observed in these controls. In addition, Table 1 and Figure 1 show several cases with an intensive staining reaction and others with a negative immunoreaction for the different markers. This provided internal negative and positive controls. For every staining procedure all 40 cases were processed simultaneously with the same standardized method. This allowed a highly reproducible comparative analysis of staining patterns and staining intensities in the different tumors.

Quantification of the Immunohistochemical Results and Statistical Analysis

The scoring was done as follows: the results of Fas and Fas-L immunohistochemistry were first classified on the percentage of positively stained cells (PPC): 10, 20, 30, 40, to 100%. Secondly, the specimens in the different groups were further classified according to the staining

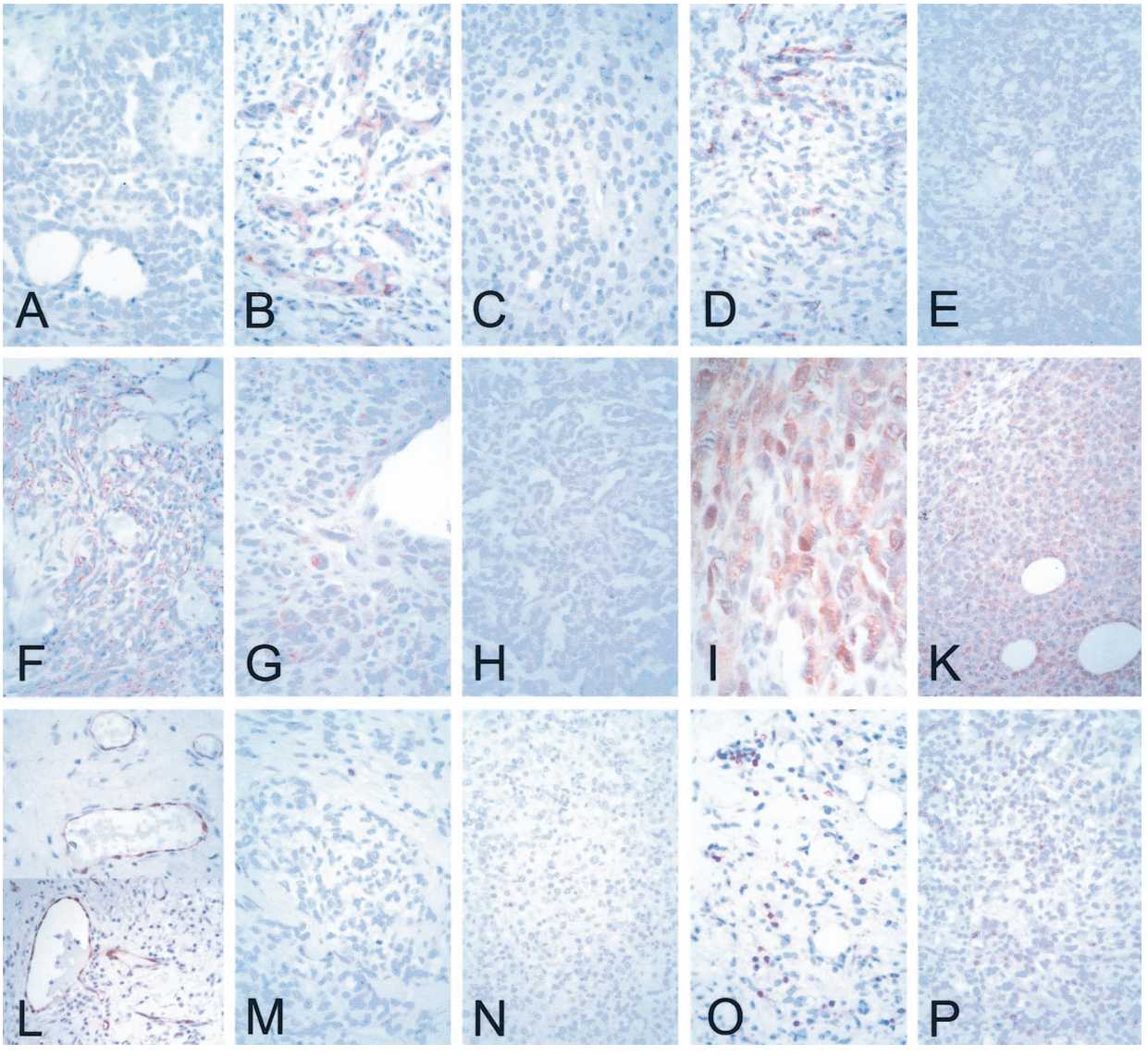

Figure 1. Immunohistochemical detection of Fas/Fas-L expression and of CD3- and CD8- positive TILs in angiosarcomas. Fas immunostaining (clone Apo-1) of AS 7 (**A**), AS 12 (**B**), (clone DX2) of AS 15 (**C**), and AS 12 (**D**). Fas-L immunostaining (clone 33) of AS 33 (**E**), AS 10 (**F**), AS 15 (**G**), (clone G247) of AS 36 (**H**), AS 5 (**I**), AS 4 (**K**), and in normal vascular endothelial cells in the peritumor area of AS 4 (**L**). Detection of CD3-positive TILs in AS 10 (**M**), AS 4 (**N**), and AS 12 (**O**). Detection of CD8-positive TILs in AS 33 (**P**). Corresponding immunostaining on the same specimens demonstrating that Fas-L expression is inversely related with TIL numbers are shown in **E** (Fas-L), **P** (CD8), **F** (Fas-L), **M** (CD3), **K** (Fas-L), and **N** (CD3), respectively. Original magnifications: ×100 (**L**); ×125 (**B**, **E**, **F**, **H**, **K**, **N**); ×150 (**D**, **O**, **P**); ×200 (**A**, **C**, **M**); ×250 (**G**); ×300 (**I**). Abbreviations: AS, angiosarcoma (see Table 1).

intensity (SI) in four categories: negative (−), low (SI-L), intermediate (SI-IM), or high staining intensity (SI-H).

The number of tumors infiltrating CD3-positive or CD8-positive T lymphocytes (TILs) was determined microscopically by evaluating vital tumor areas in 10 high-power fields (HPFs). One HPF had a field diameter of 0.5 mm and a field area of 0.196 mm^2. The mean numbers of TILs staining CD3- or CD8-positive were scored as follows: label 1, 0 to 1 positive TILs/HPF; label 2, 2 to 5 positive TILs/HPF; label 3, 6 to 10 positive TILs/HPF; label 4, >10 TILs/HPF.

The Spearman rho significance test, the Kruskal-Wallis one-way analysis test, the Mann-Whitney *U* test, the paired chi-square McNemar's test, the Kaplan-Meier method, and the log-rank test were applied in statistical analysis using the SPSS statistical software (SPSS Inc., Chicago, IL). A probability of $P \leq 0.05$ was considered statistically significant.

Results

Detection of FAS

Fas expression in angiosarcoma was investigated with two different anti-Fas antibodies (CD95-DX2 and CD95-AP0−1; Table 1). With the CD95-APO-1 antibody 23 angiosarcomas (57.5%) were negative (Figure 1A). Four cases stained positive in up to 10% of the tumor cells (SI-L, $n = 3$; SI-IM, $n = 1$) and seven sarcomas exhibited an immunoreaction in 20 or 30% of the malignant cells

(SI-L, $n = 4$; SI-IM, $n = 2$; SI-H, $n = 1$). Six cases exhibited a CD95-APO-1 immunoreactivity in 40 to 60% of the tumor cells (SI-L, $n = 4$; SI-IM, $n = 2$; Figure 1B). With the CD95-DX2 antibody 27 of 40 angiosarcomas investigated (67.5%) did not show any immunoreactivity (Figure 1C). Six cases stained positive with a SI-L in up to 10% of the tumor cells, one sarcoma exhibited an SI-L immunoreaction in 30% of the malignant cells. Six cases were positive in >40% of the tumor cells (SI-L, $n = 3$; SI-IM, $n = 2$; SI-H, $n = 1$) (Figure 1D). The two different anti-Fas antibodies produced similar results. Comparison of Fas-positive and -negative specimens obtained with both antibodies using the paired chi-square McNemar test did not show a significant difference ($P = 0.344$). Comparison of the sarcomas with negative or low Fas expression (PPC, 0 to 30%) and the tumors with high Fas expression (PPC, >30%) showed identical results in 36 cases (90%).

Detection of FAS Ligand

Fas-L expression in angiosarcoma was investigated with two different anti-Fas-L antibodies (clone 33 and clone G247; Table 1). With the first antibody (clone 33) Fas-L was not detected in 6 angiosarcomas (15%) (Figure 1E) and 13 cases (32.5%) revealed a granular-staining reaction in up to 30% of the tumor cells (SI-L, $n = 11$; SI-IM, $n = 2$) (Table 1). In all other cases (52.5%) 40 to 80% of the malignant endothelial cells were found to be Fas-L-positive (SI-L, $n = 5$; SI-IM, $n = 9$; SI-H, $n = 7$) (Figure 1, F and G). The number of Fas-L-positive cells increased significantly from grade 1 (median PPC, 20%; Table 1) to grade 3 angiosarcomas (median PPC, 70%; Table 1). With the second antibody (clone G247) Fas-L was not detected in 11 angiosarcomas (27.5%) (Figure 1H) and 5 cases (12.5%) revealed a staining reaction in up to 30% of the tumor cells (SI-L, $n = 4$; SI-IM, $n = 1$) (Table 1). In all other cases ($n = 24$) 40 to 80% of the malignant endothelial cells were found to be Fas-L-positive (SI-L, $n = 6$; SI-IM, $n = 11$; SI-H, $n = 7$) (Figure 1, I and K; Table 1).

Normal endothelium of tissue adjacent to sarcoma infiltration stained only faintly and inconsistently with the Fas-L antibody clone 33 (data not shown), whereas staining intensity with the G247 antibody was clearly higher in vessels in the peritumor area but gradually decreased in more distant vessels (Figure 1L).

Comparsison of the results obtained with two different antibodies on a qualitative basis (Fas-L-positive *versus* Fas-L-negative specimens) using the paired chi-square McNemar test did not show a significant difference ($P = 0.227$). Comparison of the sarcomas with negative or low Fas-L expression (PPC, 0 to 30%) and the tumors with high Fas-L expression (PPC, >30%) showed identical results in 29 cases, in 4 cases more cells reacted with the clone 33 anti-Fas-L antibody and in 7 cases higher numbers of positive cells were detectable with the clone G247 anti-Fas-L antibody (paired chi-square McNemar test, $P = 0.549$). Only in 5 of 40 cases (12.5%) the percentage of positive cells detected with the two different anti-Fas-L antibodies was different by >30%.

Detection of CD3- and CD8-Positive TILs

Only low numbers of CD3-positive lymphocytes (0 to 1 cells per HPF) were detected in 20% of angiosarcomas ($n = 8$) (Figure 1M–N; Table 1). In 37.5% of the sarcomas ($n = 15$) up to five CD3-positive TILs per HPF were found. Five tumors (12.5%) exhibited between 6 to 10 CD3-positive TILs per HPF and 30% of the tumors ($n = 12$) were infiltrated 10 to 40 CD3-positive TILs per HPF (Figure 1O). The median range of CD3-positive cells in angiosarcomas was 2 to 5 TILs per HPF (Table 1).

CD8-positive lymphocytes were present in low numbers (0 to 1cells per HPF) in 55% of the angiosarcomas ($n = 22$). In 5% of the sarcomas ($n = 2$) up to five CD8-positive TILs per HPF were found. Ten percent of the angiosarcomas ($n = 4$) exhibited 6 to 10 CD8-positive TILs per HPF and 30% of the tumors ($n = 12$) were infiltrated by 10 to 32 CD8-positive TILs per HPF (Figure 1P). The median range of CD8-positive lymphocytes in angiosarcomas was 0 to 1 TIL per HPF (Table 1).

Both, CD3- and CD8-positive TILs were present in significantly higher numbers in the grade 1 angiosarcomas (median CD3, >10 TILs/HPF; median CD8, 6 to 10 TILs/HPF; Table 1) as compared to grade 3 tumors (median CD3, 2 to 5 TILs/HPF; median CD8, 0 to 1 TIL/HPF; Table 1).

Correlation Analysis of Fas/Fas-L Expression and TIL Numbers

No significant difference was observed in the reactivity of the two different anti-Fas-antibodies (McNemar test, $P = 0.344$). Fas expression did not correlate with the expression of Fas-L, the numbers of CD3-positive or CD8-positive TILs, the histopathological tumor grade (Table 2), and the time of survival (CD95-Apo-1, $P = 0.1006$; CD95-DX2, $P = 0.1303$; log-rank test). Also, the two different anti-Fas-L antibodies revealed similar reactivity (Mc Nemar test, $P = 0.227$). However, in contrast to Fas expression a highly significant inverse relation of the numbers of Fas-L-expressing cells and the numbers of CD3-positive (Spearman rho rank correlation, $P < 0.001$; Table 2) and CD8-positive TILs ($P = 0.004$, Table 2) was observed. This inverse correlation of Fas-L expression and TIL numbers in angiosarcomas could be further confirmed by the Kruskal-Wallis test [Fas-L (clone 33), CD3-positive TILs, $P < 0.001$; CD8-positive TILs, $P = 0.001$; Fas-L (clone G247), CD3-positive TILs, $P = 0.004$; CD8-positive TILs, $P = 0.016$)] and the Mann-Whitney U-Test [TIL low *versus* TIL high; Fas-L (clone 33), CD3-positive TILs, $P < 0.001$; CD8-positive TILs, $P < 0.001$; Fas-L (clone G247), CD3-positive TILs, $P = 0.015$; CD8-positive TILs, $P = 0.002$]. Furthermore, TIL numbers correlated inversely with increasing histopathological tumor grades of the angiosarcomas [Spearman rho rank correlation: CD3-positive TILs, $P = 0.001$; CD8-positive TILs, $P = 0.021$ (Table 2); Kruskal-Wallis test: CD3-positive TILs, $P = 0.008$; CD8-positive TILs, $P = 0.072$].

Only with one of the anti-Fas-L antibodies (clone 33) a positive correlation of the numbers of Fas-L-positive cells

Table 2. Spearman Rank Correlation Tests

	CD 8	CD3	FAS-L (clone 33)	Fas-L (clone G 247)	CD95/Fas (clone Apo-1)	CD95/FAS (clone DX2)	Grading
CD 8							
Rank correlation r =		.741	−.628	−.443	.049	−.165	−.363
Significance $P <$		.000	.000	.004	.764	.309	.021
CD3							
Rank correlation r =	.741		−.689	−.538	.093	−.077	−.494
Significance $P <$	.000		.000	.000	.568	.635	.001
FAS-L (clone 33)							
Rank correlation r =	−.628	−.689		.522	−.046	.053	.425
Significance $P <$	.000	.000		.001	.778	.746	.006
Fas-L (clone G 247)							
Rank correlation r =	−.443	−.538	.522		−.094	.119	.151
Significance $P <$	.004	.000	.001		.562	.465	.352
CD95/Fas (clone Apo-1)							
Rank correlation r =	.049	.093	−.046	−.094		.590	−.250
Significance $P <$	.764	.568	.778	.562		.000	.120
CD95/FAS (clone DX2)							
Rank correlation r =	−.165	−.077	.053	.119	.590		−.055
Significance $P <$	.309	.635	.746	.465	.000		.734
Grading							
Rank correlation r =	−.363	−.494	.425	.151	−.250	−.055	
Significance $P <$	.021	.001	.006	.352	.120	.734	

Correlation analysis of the different results obtained in this study has been performed with the Spearman rho rank correlation test. Shown are the values of the rank correlation (r) and the significance −two-tailed (P). A value of $P \leq 0.05$ was considered statistically significant.

and increasing tumor grades was observed [Spearman rho rank correlation, $P = 0.006$ (Table 2); Kruskal-Wallis test, $P = 0.029$]. With this antibody the highest numbers of Fas-L-expressing cells (PPC >70%) were almost exclusively associated with tumor grade 3 (Table 1).

Preliminary review of the patient data in Table 1 suggested that Fas-L positivities greater than *versus* lower than 70% PPC may be associated with different survival probabilities of the patients. In fact, Kaplan-Meyer curves confirmed that survival time is shorter in patients with high Fas-L-expressing tumors (PPC >70%) as compared to those with low Fas-L-expressing tumors (PPC <70%) (Figure 2). The differences in survival times were found to be of high statistical significance for the results obtained with the clone 33 anti-Fas-L antibody (log rank test, $P = 0.0083$) and of borderline statistical significance with the clone 247 anti-Fas-L antibody (log rank test, $P = 0.0586$). Considering the numbers of cases tested, both results clearly indicate that high Fas-L expression in angiosarcomas is associated with shorter survival times of the patients.

Discussion

The Fas/Fas-L system is a key component in the regulation of apoptosis during lymphocyte development, cell-mediated immunological control of virus infection, and tumor growth. Consequently, the role and molecular function of the Fas/Fas-L system has been extensively studied in immune cells. However, recent findings suggest that Fas and Fas-L may also play an important role in the physiological function of endothelial cells: first, Fas and Fas-L are both expressed in normal ECs, both in culture and in tissues. However, this does not result in the induction of apoptosis in these cells.[10,11,15] Second, Fas-L expressed on the EC surface has been shown to inhibit extravasation of immune cells into the tissues through the induction of apoptosis in Fas-expressing leukocytes adhering to the vessel wall.[10–13] The resistance of ECs to Fas-mediated cell killing and the capability of ECs to induce apoptosis in Fas-bearing leukocytes may also contribute to the development of endothelial cell-derived malignancies including angiosarcoma, and may effect the efficacy of antitumor treatment regimens.

Here we investigated the expression of Fas and its ligand in different grade angiosarcomas. More than 50% of the tumors investigated did not express Fas. Furthermore, in the Fas-positive tumors the numbers of Fas-expressing cells were highly variable and did not correlate with any other parameter investigated in this study. These data indicate that Fas may not trigger malignancy and clinical outcome of the disease. However, absence of Fas expression and/or potential intrinsic resistance to Fas-mediated apoptosis of the tumor cells may result in high failure rates of treatment regimens that are directed to the induction of apoptosis in angiosarcoma through Fas.

Fas-L expression, in contrast to Fas, was found in more than half of the angiosarcomas. Fas-L expression has also been detected in several other malignancies including melanomas, sarcomas, and carcinomas.[23–32] In these studies it has been suggested that Fas-L-expressing tumor cells may induce apoptosis in Fas-expressing TILs and through this may evade cell-mediated tumor defense mechanisms. Here we provided evidences that this so-called Fas counterattack may also be active in angiosarcomas. Significantly reduced numbers of CD3-positive and CD8-positive TILs were detected in high Fas-L-expressing angiosarcomas as compared to specimens where Fas-L expression was low. These results suggest an important role of Fas-L in angiosarcoma by

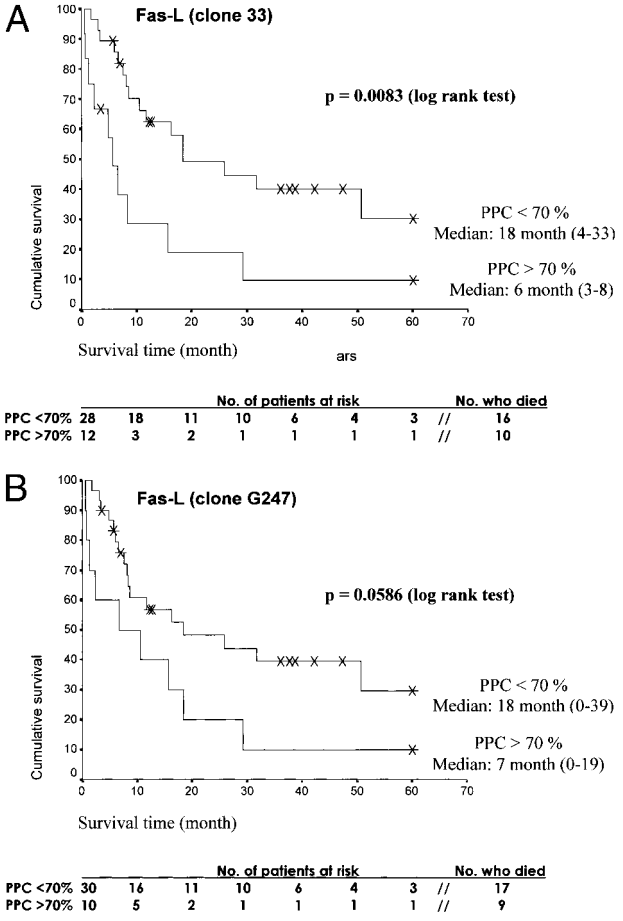

Figure 2. Kaplan-Meyer survival curves for Fas-L expression in angiosarcomas. **A:** Antibody clone 33. The median survival time (limited to 5 years) was 18 months (95% confidential interval, 4 to 33 months) for patients with a Fas-L expression in <70% of their tumor cells and 6 months (95% confidential interval, 3 to 8 months) for angiosarcomas with highest Fas-L expression. Log-rank test: $P = 0.0083$. **B:** Antibody clone G247. The median survival time (limited to 5 years) was 18 months (95% confidential interval, 0 to 39 months) for patients with a Fas-L expression in <70% of their tumor cells and 7 months (95% confidential interval, 0 to 19 months) for angiosarcomas with highest Fas-L expression. Log-rank test: $P = 0.0586$. Patients who are still alive and did not experience a sarcoma recurrence are censored (marked with X in the curves) at the date they were last seen free of clinical disease.

blocking cell-mediated anti-tumor responses. This mechanism in combination with an impairment of the p53/MDM-2 pathway previously described by us, may promote the progression of angiosarcoma.[18] The high clinical importance of Fas-L in angiosarcoma as has been described here, was clearly supported by the fact that high expression of Fas-L correlated with reduced survival times of the patients. Therefore, inhibition of Fas-L activity in angiosarcomas may open new avenues to support cell-mediated anti-tumor response and to increase the survival times of involved patients.

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