Functional characterization of Fas ligand on tumor cells escaping active specific immunotherapy

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Abstract

Mice transgenic for the rat HER-2/neu oncogene (rNeu-TG) developed spontaneous breast tumors that can escape a rNeu-specific immune response induced by active specific immunotherapy (ASI). The ability of these escape tumors to grow appeared to be due to upregulation of the Fas ligand (Fas-L) molecule. In an effort to develop tools for the better elucidation of the role of Fas-L and other regulatory mechanisms in tumor escape, we established cell lines derived from escape tumors. These tumor cell lines retained MHC class I, rNeu and Fas-L expression in vitro and formed tumors in vaccinated mice. Tumor growth was accompanied by permanent Fas-L expression in vivo, both in vaccinated and control vaccinated mice, indicating that these cells have acquired constitutive Fas-L expression. Moreover, these cells induced target cell apoptosis in vitro. Thus, these cells represent a unique tool to elucidate the importance of Fas-L expressed by tumors that escaped efficient systemic immune responses. Cell Death and Differentiation (2001) 8, 687–695.

Keywords: HER-2/neu; tumor cell lines; immune escape; Fas ligand

Abbreviations: ASI, active specific immunotherapy; TAAG, tumor-associated antigens

Introduction

The identification of tumor-associated antigens (TAAg) expressed on tumor cells has allowed the development of active specific immunotherapy (ASI) protocols. TAAg-specific immune responses have demonstrated rejection and/or regression of various established tumors in animal models. However, numerous studies have indicated that tumors can develop passive and/or active escape mechanisms to evade the immune system in immunized animals.[1] Loss of TAAg, downregulation of MHC molecules,[2–4] lack of costimulatory molecules[5–7] and upregulation of immune-suppressive factors have been reported.[8–10]

Recent reports have identified Fas (CD95, APO-1) ligand (Fas-L) as a molecule participating in tumor resistance to the immune system. Human tumors such as melanoma,[9] hepatoma,[11] gastro-intestinal cancer,[10,12,13] lung cancer[14] and breast carcinoma[15–18] have been shown to express Fas-L and to induce apoptosis of T cells in vitro. Apoptosis of tumor infiltrating lymphocytes was found in areas of Fas-L expressing tumor cells.[12] These observations led to the hypothesis of tumor counterattack by which Fas-L expressing cancers might delete tumor-specific lymphocytes and promote evasion from an anti-tumor immune response.[19,20] In contrast, other reports demonstrated that cells engineered to express high levels of Fas-L did not confer immune privilege but rather induced a pro-inflammatory granulocytic infiltration that resulted in rapid elimination of the transplanted cells.[21–24] These contradictory results indicated that the role of Fas-L to promote tumor escape in vivo needed to be further validated. In particular, no study so far has conclusively demonstrated that a tumor constitutively expressing Fas-L could delete specific anti-tumor lymphocytes and escape vaccination-induced immune rejection.

Transgenic mice (rNeu-TG) in which the rat oncogene HER-2/neu (rNeu) is overexpressed in the mammary gland epithelium develop spontaneous breast tumors after a first pregnancy.[25] We have previously shown that a vaccine consisting of allogeneic fibroblasts overexpressing rNeu (Balbc/neu) prevented spontaneous tumor formation while late escape tumors developed in some vaccinated mice.[26] Our data further showed that escape tumors upregulated Fas-L in mice with a persistent rNeu-specific systemic immune response and indicated that Fas-L expression promoted T cell apoptosis in situ and enabled tumor escape.[27]

Tumor cell lines represent a widely used tool to investigate the role of Fas-L in tumor escape. Tumor rejection was mainly observed when Fas-L-transfected cells were used and suggested Fas-L (over) expressing cells to be responsible for non-specific immunologic reactions such as the documented granulocytic infiltrations.[21–24] However, cell lines derived from Fas-L-expressing escape tumors may offer more reliable models to assess Fas-L involvement in tumor escape from ASI. We established and characterized here tumor cell lines derived from Fas-L[+] escape tumors that developed in vaccinated rNeu-TG mice. We showed that these cell lines maintained rNeu, MHC class I and Fas-L expression in vitro, formed tumors in Balbc/neu vaccinated mice and induced Fas-L-dependent target cell apoptosis in vitro. Thus, these cell lines represent a unique tool to investigate the mechanism of Fas-L-dependent tumor escape after ASI in vivo.

Results

Tumor cells derived from escape tumors maintain their ability to grow in vaccinated mice

We investigated whether tumor cells that escaped from an efficient immune response after ASI would maintain their tumor formation capacity after transplantation into vaccinated mice. Cell suspension from one representative escape tumor (mouse #241) was reinjected in vaccinated mice as shown in Figure 1. Escape tumor cells formed tumors at the injection sites irrespective of whether transplanted in mice vaccinated with control allogeneic fibroblasts (Balbc/mock) or with fibroblasts transfected with rNeu (Balbc/neu).[26] These escape properties were maintained when newly formed tumors (from mouse #490) were passaged into a Balbc/neu vaccinated mouse (mouse #557). In contrast, Balbc/neu vaccinated mice repetitively rejected a challenge with a cell suspension of spontaneous tumor cells derived from an unvaccinated mouse (e.g. mouse #345), although these cells remained tumorigenic in Balbc/mock vaccinated mice. We have previously shown that most escape tumors expressed Fas-L on their cell surface.[27] To investigate whether Fas-L expression was maintained in passaged escape tumors we assessed Fas-L expression by immunohistochemical staining. All but one (#491) tumors that developed from escape tumor #241 expressed significant levels of Fas-L. Identical results were obtained with different transplanted escape tumors (not shown). In contrast, spontaneous tumors transplanted in Balbc/mock vaccinated mice did not upregulate Fas-L expression. These results indicated that escape properties and Fas-L expression were maintained after transplantation of escape tumors in vaccinated mice.
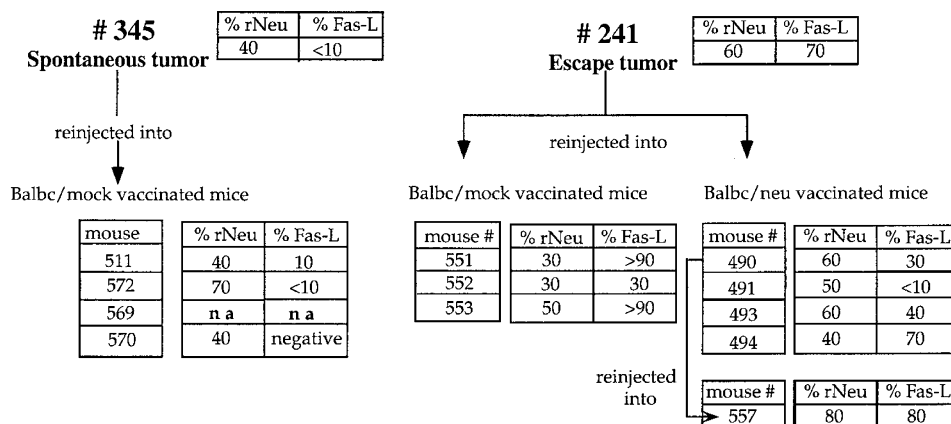
Phenotypic characterization of an escape tumor cell line

To further assess the function of naturally occurring Fas-L on tumor cells that escaped ASI, we generated stable cell lines

from spontaneous and escape tumors. Two representative cell lines derived from a spontaneous (NF9006) and as escape tumor (#493) were further analyzed. Both tumor cell lines grew in vitro as a monolayer with an epithelial-like morphology and stained positive for pan-cytokeratin (not shown). As depicted in the FACS histograms in Figure 2A, NF9006 and #493 cells expressed similar levels of rNeu. In contrast, the #493 escape tumor cell line expressed higher levels of MHC class I (H-2D^{q}L^q, IgG2a) molecules. Both NF9006 and #493 cell lines were negative for MHC class II (I-A^q, IgG2a isotype-matched control) and for CD80 and CD86 (not shown). Fas-L surface expression was assessed using a mAb recognizing the extracellular part of murine Fas-L (IgG2a). The escape tumor cell line #493 expressed elevated levels of Fas-L on the cell surface in comparison to the weak Fas-L levels on NF9006 cells. Previous studies have reported inconsistency in Fas-L detection by flow cytometric analysis depending on the mAb used.[28] To further confirm increased Fas-L protein production in #493 cells, immunohistochemical staining was performed on cytospins using a rabbit polyclonal antibody against the intracellular part of Fas-L (Figure 2B). The spontaneous tumor cell line NF9006 showed only a faint staining for Fas-L while significant Fas-L protein level was detected in the cytoplasm and membrane of #493 cells. We also performed RT−PCR for Fas-L on total RNA extracted from NF9006 and #493 tumor cells. No fragment was amplified from RNA extracts without reverse transcriptase ruling out DNA contamination. The resulting PCR product was the expected size of 547 bp (data not shown) and showed an identical sequence as the reported murine Fas-L.[29]

The escape cell line #493 maintains the capacity of tumor formation in Balbc/neu vaccinated mice

We then investigated whether the #493 tumor cell line expressing Fas-L and cultured in vitro maintained its escape capacity in vivo. NF9006 and #493 cells ($0, 5-2 \times 10^6$) were injected into Balbc/mock or Balbc/neu-vaccinated mice and

#345
Spontaneous tumor

% rNeu	% Fas-L
40	<10

#241
Escape tumor

% rNeu	% Fas-L
60	70

reinjected into

reinjected into

Balbc/mock vaccinated mice

Balbc/mock vaccinated mice

Balbc/neu vaccinated mice

mouse		% rNeu	% Fas-L
511		40	10
572		70	<10
569		n a	n a
570		40	negative

mouse #		% rNeu	% Fas-L
551		30	>90
552		30	30
553		50	>90

mouse #		% rNeu	% Fas-L
490		60	30
491		50	<10
493		60	40
494		40	70

reinjected into

mouse #		% rNeu	% Fas-L
557		80	80

Figure 1 Cell lines derived from escape tumors maintain the escape properties of their originating tumor. Cell suspensions (2×10^6) derived from spontaneous breast tumor #345 or from escape breast tumor #241 were injected s.c. into Balbc/mock or Balbc/neu vaccinated mice, as indicated. Tumor formation was monitored at the injection site. Where indicated, 2×10^6 tumor cells derived from mouse #490 was reinjected into a Balbc/neu vaccinated mouse. The results of r-Neu and Fas-L expression (per cent of positive cells), as assessed by three independent immunohistochemical stainings with specific Abs are shown for each tumor. (na, not assessed)

time to tumor formation and kinetics of tumor growth were assessed. As shown in Table 1, tumors formed in 12 out of 15 Balbc/neu vaccinated mice challenged with #493 escape tumor cells. There was no significant difference in the time to

tumor formation between Balbc/mock (mean 23.8 days, S.E.M. 3.6) and Balbc/neu (mean 31.2 days, S.E.M. 4.2, $P < 0.28$) vaccinated mice. The growth kinetic was similar in Balbc/neu and Balbc/mock vaccinated mice showing in each
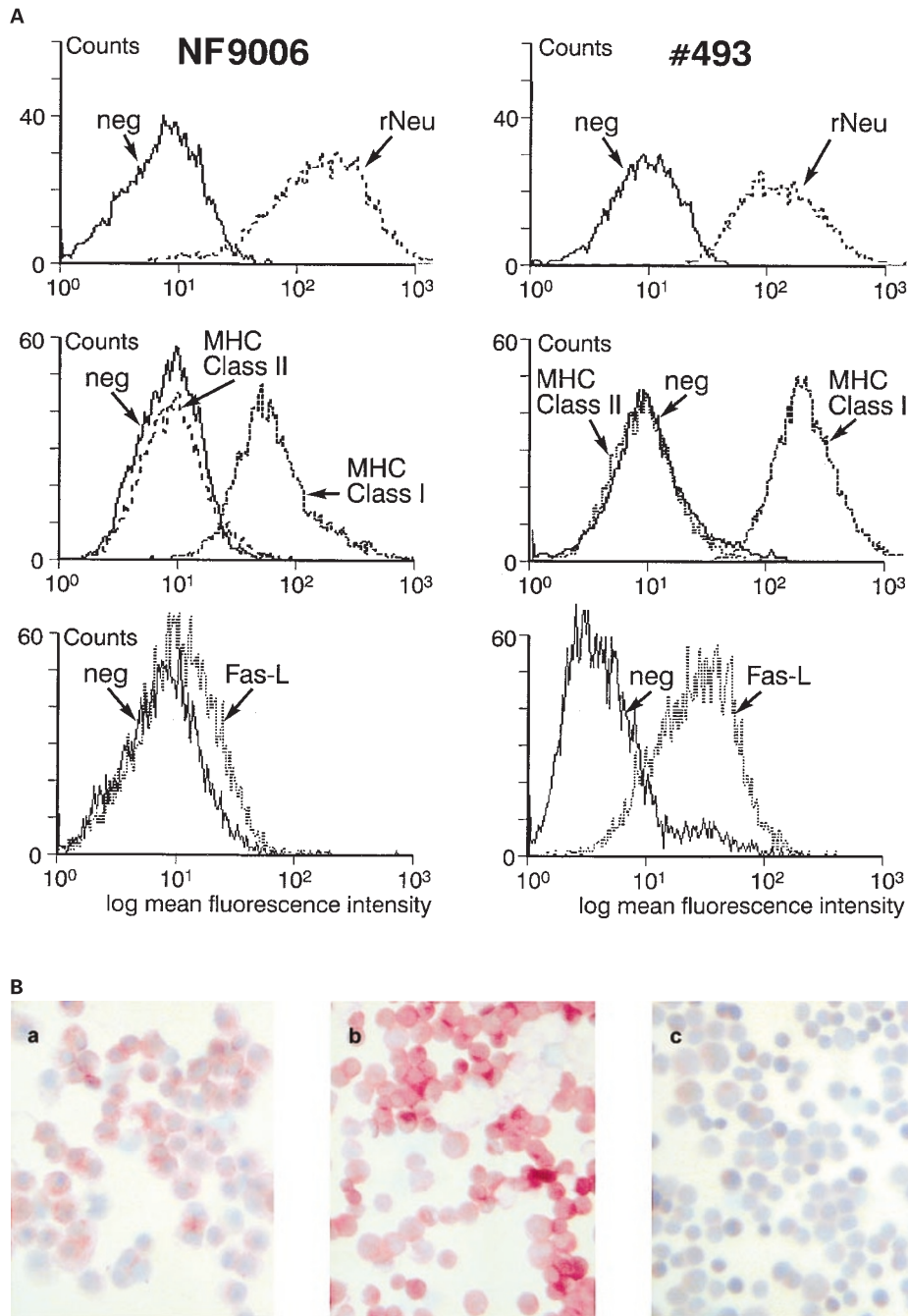
Figure 2 Analysis of Fas-L expression in NF9006 and #493 tumor cell lines. (**A**) rNeu[+] spontaneous tumor cell line NF9006 and escape tumor cell line #493 were analyzed for rNeu, MHC class I (H-2D^q/L^q), MHC class II (I-A^q) and Fas-L cell surface expression by indirect immunostaining using specific Abs followed by Streptavidin-FITC or FITC-conjugated goat anti-mouse IgG. Control staining (neg) was performed in the absence of corresponding primary Ab. Isotype-matched (IgG2a) control antibody is anti-I-A^q (MHC class II). Fluorescence was analyzed on a FACScan. (**B**) Cytospins of NF9006 (a) and #493 (b) cell lines were analyzed for Fas-L expression by immunohistochemical staining using a Fas-L specific Ab, as described in Materials and Methods. Also shown is a control staining of #493 cell line (c) using a rabbit anti-Fas primary Ab

group nine mice with tumors below 1.5 cm^2 at day 25 (Figure 3). In contrast, nine of 10 mice vaccinated with Balbc/mock exhibited a tumor size of $>$1.5 cm^2 50 days after challenge, whereas at this time point four Balbc/neu vaccinated mice had tumors $<$1.5 cm^2 and three mice showed still no tumor at the injection site. At day 50, mice vaccinated with Balbc/mock

Table 1 Time to tumor formation of injected escape tumor cell lines in vaccinated mice

Injected escape tumor cells #493	Time to tumor formation (days)	
	Balbc/mock vaccinated mice	Balbc/neu vaccinated mice
0.5 × 10^6 cells	12	24
	30	30
	35	26
		No tumor formation
1 × 10^6 cells	12	17
	38	16
	42	16
		18
		43
2 × 10^6 cells	12	20
	19	30
	19	24
	19	24
		No tumor formation
		No tumor formation

0.5-2 × 10^6 #493 tumor cells were injected s.c. into Balbc/mock or Balbc/neu vaccinated mice, as indicated, and tumor growth was monitored at the injection site. Results are expressed as time to first detection of palpable/visible tumor mass

fibroblasts showed a mean tumor size of 3.12 cm^2 (S.E.M. 0.71), whereas mice vaccinated with Balbc/neu cells had a mean tumor size of 1.53 cm^2 (S.E.M. 0.3). Tumors formed in mice irrespective of whether they were injected with 0.5, 1 or 2 × 10^6 tumor cells. However there was a significant delay of tumor growth/size in Balbc/neu vaccinated mice (Mann–Whitney rank test $P<0.024$). Moreover, NF9006 formed tumors in all Balbc/mock vaccinated mice and no tumor was observed in Balbc/neu vaccinated mice as described previously.[26] These experiments showed that the #493 cells derived from an escape tumor maintained the tumorigenic and escape capacity of the originating tumor.

Injected #493 cells maintain Fas-L expression *in vivo*

To test whether Fas-L was still expressed *in vivo* in the long-term follow up, #493 and NF9006 cells were injected into Balbc/neu and Balbc/mock vaccinated mice and expression of Fas-L *in situ* was assessed by immunohistochemistry at the injection site. As shown in Figure 4A, #493 cells injected into Balbc/neu vaccinated mice formed disrupted tumor islets by day 5 and most tumor cells stained positive for Fas-L. We have previously shown that escape tumor cells injected in Balbc/neu vaccinated mice were infiltrated by a large number of CD3$^+$ lymphoid cells in the tumor implant as well as the surrounding tissue.[27] We further showed that expression of Fas-L on tumor cells correlated with local induction of T cell apoptosis as predominantly, cells in the close periphery of injected escape tumor cells, stained positive for active caspase-3. The regions of caspase-3 activity overlapped
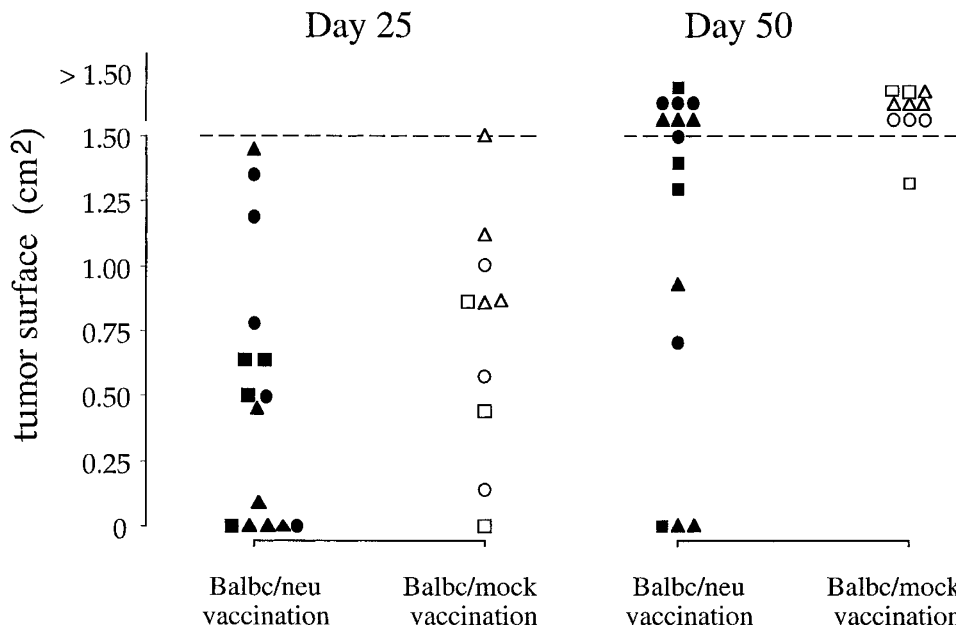
Figure 3 Tumor kinetics of injected escape tumor cell lines in vaccinated mice. Results as in Table 1 are expressed as tumor surface (cm^2) at the injection site at days 25 and 50 after injection. Open symbols are mice vaccinated with Balbc/mock fibroblasts and closed symbols are mice vaccinated with Balbc/neu fibroblasts. Challenge with 0.5 × 10^6 cells (squares), 1 × 10^6 cells (circles) and 2 × 10^6 cells (triangles)
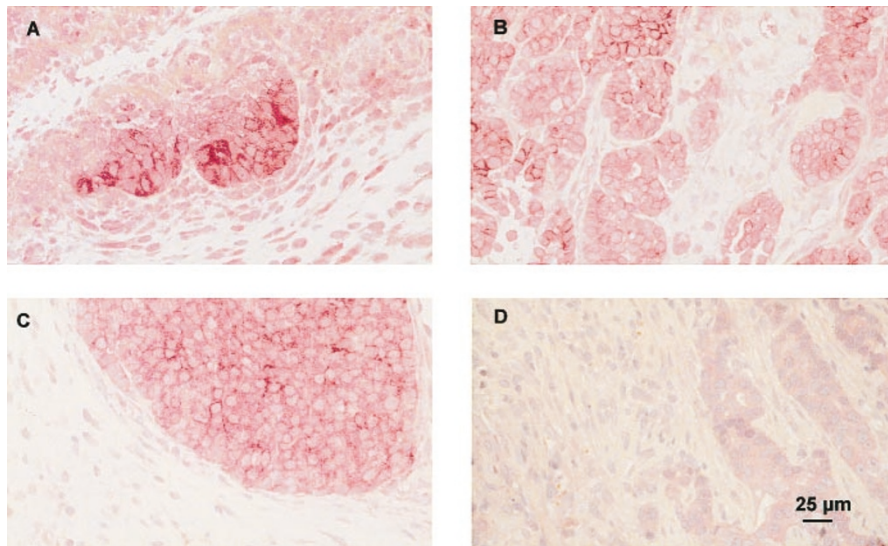
Figure 4 Expression and distribution of Fas-L in injected escape and spontaneous tumor cells. Tumors at days 5 (**A**) and 30 (**B**) after injection of #493 escape tumor cells into Balbc/neu-vaccinated mice were analyzed for Fas-L expression by immunohistochemical staining with specific Ab. Also shown are Fas-L stainings of #493 tumor cells (**C**) and NF9006 tumor cells (**D**) 30 days after injection into Balbc/mock vaccinated mice. Magnification × 40 (Bar represents 25 μm)

with the localization of CD3^{+} lymphocytes. At day 30 after challenge, tumors maintained a marked Fas-L staining (Figure 4B). No T cell infiltration was seen in close proximity of escape tumors at this time point. The same intense Fas-L expression was also detected in tumors of Balbc/mock vaccinated mice after challenge with #493 (Figure 4C). NF9006 cells that formed tumors in Balbc/mock vaccinated mice stained negative for Fas-L throughout the observation time and no tumor developed in Balbc/neu vaccinated mice challenged with NF9006 cells (not shown). These results indicated that #493 cells maintained Fas-L expression *in vivo* in the presence of a TAAg-specific immune response and that the allogeneic immune response induced by Balbc/mock cells did not cause Fas-L expression on spontaneous tumor cells.

Escape tumor cell lines induce apoptosis of Fas-L-sensitive target cells

We next investigated whether Fas-L expressed on tumor cells might be functionally responsible for the induction of apoptosis of infiltrating T cells.[27] We used the P815 mastocytoma cell line transfected with murine Fas cDNA as Fas-L-sensitive target cells in a modified cytotoxicity test.[30,31] P815-Fas cells have been used as appropriate target cells to investigate putative Fas-L activity on tumor cells, as no Fas-L is expressed on P815 wild type cells (P815-WT, Calzascia personal communication). P815-WT and P815-Fas were cultured either alone or together with NF9006 or #493 cells. The data depicted in Figure 5A demonstrated that #493 tumor cells mediated a cell density dependent killing of P815-Fas cells reaching approximately 40% lysis at E : T ratio of 16 while no DNA fragmentation was observed in P815-WT. In contrast, the spontaneous tumor cell line NF9006 did not induce significant DNA fragmentation neither of P815-Fas nor

of P815-WT cells. In addition, we observed the same dose-dependent cytotoxicity with a different escape tumor cell line (#242, data not shown). To further support the finding that Fas-L on #493 cells was responsible for DNA fragmentation of P815-Fas cells, cocultures were performed in the presence of Fas-Fc fusion protein (20 μg/ml) to block the Fas/Fas-L interaction. The data shown in Figure 5B indicated that cytotoxicity induced by #493 was mainly mediated by Fas-L as the addition of Fas-Fc fusion protein inhibited DNA fragmentation. The same inhibition of DNA fragmentation was observed when #242 escape tumor cells were used as effector cells and Fas-Fc protein was added to the culture system (data not shown). In contrast, Fas-Fc fusion protein had no effect on cell cultures with P815-WT or in coculture systems with NF9006 as effector cells. Thus, the capacity of Fas-L expressing #493 cells to kill Fas-sensitive target cells and its inhibition by Fas-Fc fusion protein strongly suggests a role of constitutively expressed Fas-L on tumor cells as an active tumor escape mechanism.

Discussion

We have previously shown that some rNeu-TG mice developed late escape mammary tumors after a rNeu-specific vaccination with Balbc/neu fibroblasts.[27] These escape tumors expressed elevated levels of surface Fas-L that appeared to promote tumor escape from rNeu specific immune response after vaccination. This created a unique opportunity for the isolation of cell lines that constitutively expressed Fas-L. We showed here that cell lines derived from rNeu[+] escape tumors maintained the Fas-L expression of the original tumor and were tumorigenic in Balbc/neu vaccinated mice, in contrast to a tumor cell line derived from a spontaneous tumor that developed in unvaccinated mice.
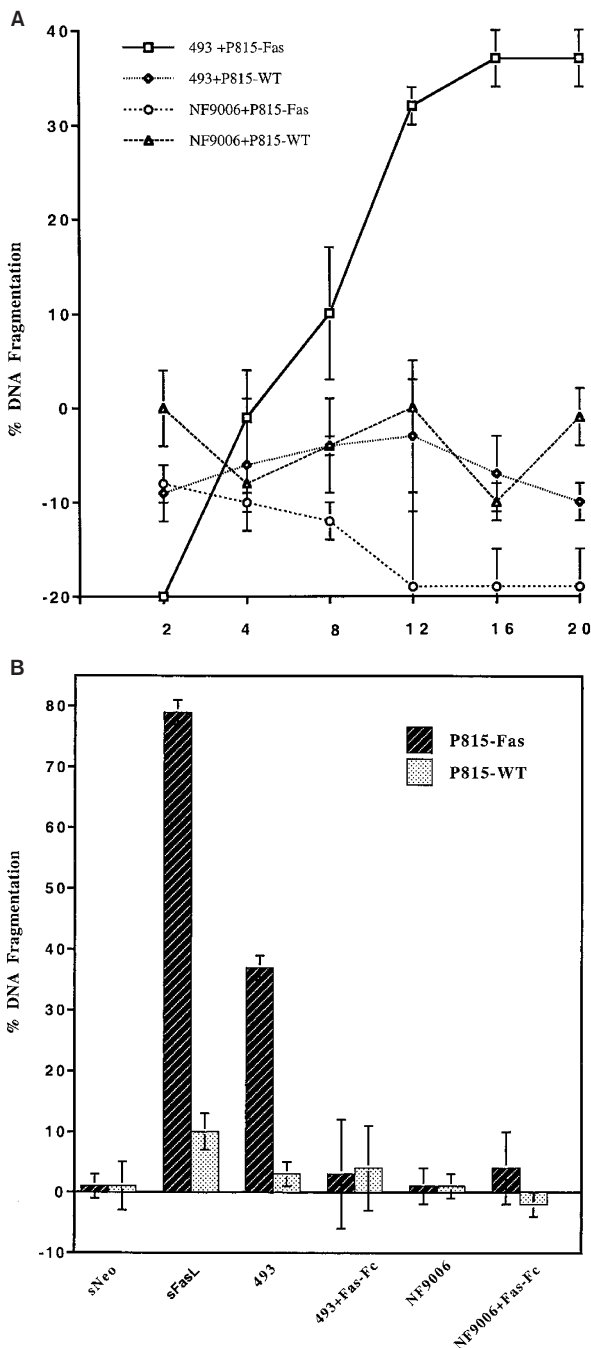
Figure 5 Fas-L expressed on #493 escape cells mediates DNA fragmentation in Fas[+] P815 mastocytoma cells. (**A**) P815-Fas and P815-WT cells were labeled with ^{3}H-thymidine and used as targets to study the ability of #493 escape tumor cells to mediate cell death in comparison to the spontaneous tumor cell line NF9006. Target cells were used at a concentration of 5000 cells/well. Different effector: target ratios (E : T) were investigated as indicated. Results are expressed as % DNA fragmentation in relation to target cells in the absence of effector cells. Vertical bars represent mean ± s.d. in triplicate cultures. (**B**) ^{3}H-thymidine labeled P815-Fas and P815-WT cells (5000 cells/well) were cultured *in vitro* for 8 h in the presence of Neo supernatant (sNeo, 1 : 4=v : v), supernatant from Neuro-2a cells stably transfected with Fas-L (sFas-L, 1 : 4=v : v), or cocultured with 60 000 adherent #493 escape or NF9006 spontaneous tumor cells. Fas-Fc fusion protein was added at a concentration of 20 μg/ml as indicated. Results are expressed as in **A**. This is one representative experiment of three performed with similar results

Moreover, we observed Fas-L expression throughout the *in vivo* tumor growth, both in Balbc/mock and Balbc/neu vaccinated mice. Escape tumor cell lines induced a Fas-dependent target cell apoptosis *in vitro*, thus demonstrating the functional activity of cell surface Fas-L.

Fas-L plays a pivotal role in lymphocyte cytotoxicity and in the maintenance of immunologic homeostasis[32] and has been implicated in immune privilege of various organs, such as the eye and testis by inducing apoptosis of activated T lymphocytes.[33,34] Immune privilege has also become a new focus in tumor immune evasion, as various types of cancer cells including breast cancer,[15–18] hepatocellular carcinomas,[11] colon cancer[10] and melanoma[9] have been described to express Fas-L. The functional activity of tumor-derived Fas-L was demonstrated *in vitro* by the induction of apoptosis of activated T cells and *in vivo* by the detection of apoptotic T cells in the vicinity of Fas-L expressing tumors.[9–14] We have shown in our rNeu-TG mouse model a predominant expression of Fas-L on escape tumor cells. We further demonstrated here that Fas-L also expressed on a representative escape tumor cell line that formed tumors in vaccinated mice. Moreover, we previously described apoptotic T cells in close proximity of Fas-L expressing escape tumors, in contrast to Fas-L negative spontaneous tumors.[27]

However, the initial observations of Fas-L expression on melanoma cells and the induction of apoptosis of activated T cells[9] were not confirmed by subsequent studies,[21,35] putting Fas-L in question as a tumor counterattack mechanism.[20,36] Fas-L detection on some tumor samples has suffered from technical inconsistencies based on unspecific Abs,[28,37] and the lack of Fas-L detection using FACS or RT–PCR.[21,35,38] We demonstrated Fas-L expression on escape tumor cell lines by FACS analysis, thus avoiding lymphocyte contamination that may contribute to Fas-L detection in fresh tumor samples, by immunohistochemistry using a different antibody both on cytospins and on tumor samples after reinjection *in vivo* and by RT–PCR using specific primers and sequencing of the resulting fragment.

Further controversies arose when transplanted cells, engineered to express Fas-L either by transgene expression,[22,23] by transfection[24] or by transduction with an adenoviral vector,[21] were shown to exhibit a proinflammatory effect that resulted in a profound infiltration of neutrophils and in tumor rejection. This immunologic reaction was explained by the apoptotic death of T cells and other Fas-expressing cells and the production of IL-1β through their activated cascade of caspase proteases or of IL-8 by endothelial cells.[39] These observations significantly differ from our results, as we failed to detect an inflammatory infiltration of neutrophils in growing escape tumors or after injection of escape tumor cells. This is consistent with our previous observation that tumor rejection was mediated by T cells[26] and that apoptotic T cells were specifically found in close proximity of Fas-L[+] escape tumor cells.[27] Moreover, we demonstrated here that Fas-L[+] escape tumor cell lines induced a Fas-dependent target cell apoptosis *in vitro* that was blocked with a soluble Fas-Fc fusion protein. These results indicate that Fas-L on

the tumor cell surface was functional and may have enabled tumor escape *in vivo*. The likely involvement of Fas-L in the escape of tumor cells is further supported by our observation that as few as 0.5×10^6 injected escape tumor cells formed tumors in vaccinated mice (see Table 1 and Figure 3), indicating that these cells were not simply outnumbering the immune response, but displayed an active immunosuppressive mechanism *in situ* to escape the systemic rNeu-specific immune response. The significant delay of escape tumor progression in Balbc/neu vaccinated mice in comparison to Balbc/mock vaccinated mice may result from the different degree of Fas-L expression on escape tumors. The faint Fas-L expression in IHC and FACS analysis of the spontaneous tumor cell line (NF9006) indicated that a Fas-L expression above a certain threshold was necessary for tumor cells to escape an ASI.

Tumor escape from immune response appears a complex mechanism that, besides Fas-L, likely involves other immunomodulatory factors, such as TGF-β.[40] The availability of escape tumor cell lines constitutively expressing both a clinically relevant TAAg (rNeu) and Fas-L may help to clarify some of the controversies surrounding the involvement of Fas-L in tumor escape mechanisms.[20,36] In particular, protocols to prevent Fas-L expression or Fas-L-mediated escape *in vivo* could be investigated. This may be of relevance in future clinical trials of ASI in patients with breast cancer, as Fas-L expression was shown in various breast carcinomas and derived cell lines.[16–18]

Materials and Methods

Mice

MMTV/r-Neu FVB mice (H-2^q) transgenic for the rat neu protein (rNeu-TG) were purchased from Charles River, Germany. Animal care was in accordance to institutional guidelines. Transgenic animals were identified by PCR for rNeu as described previously.[26]

Cell lines

Balb/c 3T3 (H-2^d) fibroblasts transfected with a SV2-Neo-SP65 plasmid alone (Balbc/mock) or together with the pBR322-rNeu plasmid (Balbc/neu) have been described previously.[26]

Establishment of spontaneous and escape tumor-derived cell lines

Tumors were excised and minced into small pieces (<0.5 mm each) in PBS and incubated for 20 min at 37°C in the presence of Trypsin/EDTA (Sigma, Buchs, Switzerland). The cell suspension was filtered through a 0.45 μm cell strainer (Becton Dickinson, Heidelberg, Germany), washed in DMEM supplemented with 10% FCS, and grown in William's E medium supplemented with 10% FCS, antibiotics (penicillin/streptomycin), 2 mM glutamin, and 10^{-5} M β-mercaptoethanol (all from Sigma). Established cell lines were then progressively adapted from William's E (Sigma) to DMEM medium. The rNeu$^+$ breast cancer cell line NF9006 was cloned from a spontaneous breast adenocarcinoma as described.[41]

Vaccination protocols

For vaccination/challenge experiments, mice were vaccinated and boosted (after 14 days) intraperitoneally (i.p.) with 5×10^6 Balbc/neu or Balbc/mock fibroblasts. Two weeks after the boost, mice were challenged by subcutaneous (s.c.) injection of $0.5 – 2 \times 10^6$ rNeu$^+$ spontaneous or escape tumor cells. Tumor formation and size was assessed every 3–4 days using a calibrator. Tissues were collected for histological and immunohistochemical analysis at indicated time points.

Immunohistochemistry

Paraffin-embedded tissues were deparaffinized and pretreated by microwave heating in 10 mM citrate buffer, pH 6.0. Slides were incubated with either a rabbit anti-HER-2/neu (Dako, Copenhagen, Denmark), a rabbit anti-Fas-L (N-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or a rabbit anti-topoisomerase IIα (TOPOIIAp, Novocastra Laboratories, Newcastle, UK) polyclonal antibody (Ab) diluted in TCGA buffer (Tris 50 mM, pH 7.5 supplemented with 1% casein, 0.02% NaN$_3$ and 5% normal goat serum). Alternatively, cytospins were incubated with a mouse anti-pan-cytokeratin (Sigma), a rabbit anti-Fas-L (N20) or rabbit anti-Fas (Oncogene Research, MA, USA) polyclonal Ab diluted in TCGA. After incubation with biotin-conjugated goat anti-mouse or goat anti-rabbit Ab (Dako), reactivities were detected using alkaline phosphatase-conjugated avidin-biotin complex (Dako) and Newfuchsin substrate (Sigma) according to manufacturer's instructions. Stainings were scored as described.[27]

FACS analysis

Cells were incubated at 4°C for 30 min with mouse anti-rNeu Ab-4 (Oncogene Science, Tarzana, CA, USA) or biotinilated anti-Fas-L (H11, IgG2a from Alexis, Lausen, Switzerland), anti-H-2D^q/L^q, anti-I-A^q, anti-CD80 or anti-CD86 mAbs (IgG2a, all from Pharmingen, San Diego, CA, USA), washed with PBS, followed by incubation with FITC-conjugated Streptavidin or FITC-conjugated goat anti-mouse IgG (Southern Biotechnology, Birmingham, AL, USA) and analyzed for fluorescence on a FACScan (Becton Dickinson, Heidelberg, Germany).

RT–PCR for Fas-L

Total RNA was extracted from 5×10^6 tumor cells using the SVTotal RNA Isolation system (Promega, Madison, WI, USA) according to manufacturer's instructions. One μg RNA was subjected to reverse transcriptase reaction (RT) using 0.5 μg oligo p(dT)$_{15}$ primer, 0.4 mM dNTP nucleotide mix (Roche Diagnostics, Rotkreuz, Switzerland) and the M-MLV reverse transcriptase (Promega) for 1 h at 42°C. Total RNA extracts without reverse transcriptase was used as control ruling out DNA contamination. RT reaction was stopped by incubation at 95°C for 5 min. cDNA was then subjected to 40 PCR cycles (1 min at 95°C, 1 min at 62°C, 1.5 min at 72°C) using intron-spanning primers: 5-CACTGACCCCTCTAAAGAAGAA-3′ (forward) and 5′-TTGAATACTGCCCCCAGGTA-3′ (reverse). Presence of a 547-bp fragment was analyzed on an agarose gel. Fas-L fragment was further confirmed by sequencing (Microsynth GmbH, Balgach, Switzerland).

Co-culture DNA fragmentation assay (JAM test)

Target Fas-transfected P815-4.2 (P815-Fas) and P815 wild type (P815-WT) cells were kindly provided by K Matsuura and P Vassalli, (Geneva, Switzerland). The cell death was quantitated by measuring

target cell DNA fragmentation using the JAM test with some modifications.[30,31] In brief, breast cancer cell lines (NF9006 and #493) were seeded in flat bottom 96-well plates and incubated overnight. Thereafter, 5000 P815-WT or P815-Fas target cells were added that had been labeled by prior incubation with 2.5 μCi/ml of [3H]thymidine at 37°C for 18–22 h. After a coculture of 6–8 h cells were removed from the wells by adding 5 mM EDTA and collected onto a glass fiber filter. The radioactivity of intact chromosomal DNA retained on filters was measured by liquid scintillation counting. Specific killing was calculated using the following equation:

$$\% \text{ Specific killing} = (S \quad E/S) \times 100$$

where E (experimental) is c.p.m. of retained DNA in the presence of effector cells, and S (spontaneous) is c.p.m. of retained DNA in target cells in the absence of effector cells. Soluble Fas-L was obtained from supernatant of Neuro-2a cells stably transfected with Fas-L as described previously[42] and kindly provided by A Fontana (Zurich, Switzerland). Where indicated Fas-Fc fusion protein was added to the cultures to inhibit Fas/Fas-L activity at a concentration of 20 μg/ml according to manufacturer's instructions (Alexis).

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