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Fas Ligand on Tumor Cells Mediates Inactivation of Neutrophils

Yi-Ling Chen,* Shun-Hua Chen,† Jiu-Yao Wang,‡ and Bei-Chang Yang2†

The expression of Fas ligand (FasL) on tumor cells (tumor FasL) has been implicated in their evasion of immune surveillance. In this study, we investigated the cellular mechanism for FasL-associated immune escape using melanoma B16F10-derived cells as a model. Transfectants carrying FasL-specific ribozymes expressed low levels of FasL (FasLlow tumor cells) as compared with those carrying enhanced green fluorescent protein-N1 plasmids (FasLhigh tumor cells). When injected s.c. into C57BL/6 mice, FasLlow tumor cells grew more slowly than did FasLhigh melanoma cells. FasLhigh tumor cells showed more intensive neutrophilic infiltration accompanied by multiple necrotizing areas than did FasLlow tumor cells. The average size of FasLlow tumors, but not of FasLhigh tumors, was significantly enhanced in mice depleted of neutrophils. Consistently, a local injection of LPS to recruit/activate neutrophils significantly delayed tumor formation by FasLlow tumor cells, and slightly retarded that of FasLhigh tumor cells in both C57BL/6 and nonobese diabetic/SCID mice. Neutrophils killed FasLlow melanoma cells more effectively than FasLhigh melanoma cells in vitro. The resistance of FasLhigh melanoma cells to being killed by neutrophils was correlated with impaired neutrophil activation, as demonstrated by reductions in gelatinase B secretion, reactive oxygen species production, and the surface expression of CD11b and the transcription of FasL. Local transfer of casein-enriched or PMA-treated neutrophils delayed tumor formation by melanoma cells. Taken together, inactivation of neutrophils by tumor FasL is an important mechanism by which tumor cells escape immune attack.


The fact that neutrophils are recruited into a FasL-positive tumor region without causing growth retardation in tumors suggests a mechanism by which tumor cells can attenuate the antitumor processes of neutrophils in situ during these encounters. Another point that remains to be answered is whether the effect of FasL on immunity observed in ectopic expression systems still holds true in other systems. This argument is implied by the finding that some FasL-positive tumors do induce a mild degree of inflammation, but which, however, fails to cause tumor rejection (28). In this study, these questions were further addressed using a functional knock-down strategy with a hammerhead ribozyme to specifically suppress the FasL gene (27). The important role of neutrophils in antitumor immunity was demonstrated by depletion experiments using Abs for CD4+ T cells, CD8+ T cells, and neutrophils. Culture experiments were used to evaluate the alterations in functional activities of neutrophils in contact with tumor cells expressing different levels of FasL. A possible antitumor therapy that attempts to activate neutrophils was also tested. The results obtained not only complement previous findings using FasL overexpression systems, but also clearly demonstrate a crucial role for neutrophils in determining tumor immunity.

Materials and Methods

Reagents and Abs

H&E, glycerol gelatin, propidium iodide (PI), LPS from Escherichia coli (O55:b5 strain), acetylcysteine, and PMA were purchased from Sigma-Aldrich (St. Louis, MO). An aminoethyl carbazole substrate kit was purchased from Zymed (San Francisco, CA). The 2′,7′-dichlorofluorescein diacetate (H2DCFDA) was purchased from Molecular Probes (Eugene, OR). The following Abs were used in this study: rat IgG Ab (ICN Pharmaceuticals, Cappel, OH); recombinant mouse FasFc chimeric protein (mFasFc; R&D Systems, Minneapolis, MN); rat anti-NK mAb (DX5), rat anti-CD4 mAb (H129.19), rat anti-CD8 mAb (53-6.7), rat anti-Ly-6G (RB6-8C5) mAb, anti-CD4 FITC (H129.19), anti-CD8 PE (53-6.7), anti-Ly-6G FITC (RB6-8C5), and anti-CD11b (M1/70, PE conjugated) purchased from BD Pharmingen (San Diego, CA); rabbit anti-FasL Ab (N-20) (Santa Cruz Biotechnology, Santa Cruz, CA); goat anti-rat IgG peroxidase.
conjugate (Chemica, San Diego, CA); and goat anti-rabbit IgG peroxidase conjugate (Calbiochem, San Diego, CA).

**Transfectants and cell culture**

The B16F10-derived melanoma cells used in this study were established, as previously reported (27). After DNA transfection followed by antibiotic selection, stable transfectants were established. Applying FasL ribozyme suppressed the expression of FasL in melanoma cells in vitro. B16F10 transfectants carrying the pEGFP (enhanced green fluorescent protein)-N1 plasmid were named Vn for FasL low tumor clones; B16F10 transfectants carrying the FasL ribozyme plasmid were named Rn for FasL high tumor clones. The B16F10-derived melanoma cells used in this study were established, as previously reported (32), and reduced expression of FasL at both the transcriptional and translational levels (27).

**Tumor formation**

Tumor cells cultured for 3 days were harvested with 0.05% trypsin-EDTA and washed twice with sterile PBS. Tumor cells were suspended in ice-cold 70% ethanol overnight at 4°C, and stained with a solution containing 5 mg/ml 1-glutamate at 5°C/37°C in a humidified atmosphere. All cell culture medium was purchased from Life Technologies (Grand Island, NY).

**Mice**

Eight-week-old C57BL/6 (H12) mice and nonobese diabetic (NOD)/SCID (lymphocyte- and NK cell-deficient) mice were purchased from the Laboratory Animal Center of National Cheng Kung University and were maintained under pathogen-free conditions. All animal experiments were conducted with the approval of the ethics committee of our institution.

**Histological examination and immunohistochemistry**

Tissues were surgically obtained and immersed in a buffered 10% Formalin solution for at least 24 h. Sections 4 μm thick were dehydrated, embedded, and stained with H&E. The tissues for immunohistochemical staining were embedded in OCT compound (Miles, Elkhart, IN), frozen in liquid nitrogen, and stored at −20°C. Five-micrometer-thick cryosections were placed on poly(t-L-lysine)-coated glass slides and fixed with 3.7% formaldehyde in either PBS for 15 min plus acetone for 3 min or 3.7% paraformaldehyde for 5 min plus acetone for 1 min. The endogenous peroxidase activity in tissue sections was depleted by incubation in PBS containing 5% H2O2 for 30 min. The primary Ab was diluted with Ab diluent (DAKO, Carpenteria, CA), and included rat anti-NK mAb (DX5), rat anti-CD4 mAb (H12.19.2), rat anti-CD8 mAb (53-6.7), rat anti-Ly-6G (RB6-8C5) mAb, or rabbit anti-FasL (N-20). The secondary Abs were sheep anti-rat IgG conjugate or goat anti-rabbit IgG peroxidase. Redish-brown peroxidase staining was developed with an aminochel carbazole substrate kit. Sections were counterstained with hematoxylin and mounted in glycerol gelatin.

**Apoptosis**

Tumor cells or neutrophils were analyzed for apoptosis by a PI analysis that detected hypodiploid nuclei. Cells that appeared in sub-G1/G0 peaks were apoptotic. In brief, cells were harvested, suspended in ice-cold 70% ethanol overnight at 4°C, and stained with a solution containing 5 μg/ml of PI (30). In neutrophil and tumor cell co-cultures, viable tumor cells were distinguished from neutrophils or dead tumor cells by side and forward scatter parameters. In situ detection of apoptosis was performed with TUNEL staining. Cytospin cells were stained using a commercially available kit (ApoTag in situ apoptosis detection kit-peroxidase; Intergen, Purchase, NY), following the manufacturer's instructions. The apoptotic cells were examined with conventional light microscopy at ×400 to evaluate tumor cells, and 100 cells were counted per sample. Results shown are from individual mice, and data were reported as the percentage of cells with apoptotic morphology.

**Isolation of mouse neutrophils from peritoneal fluid**

Mouse neutrophils in peritoneal fluid were isolated according to the procedures described previously (33). In brief, about 3–5 × 107 peritoneal exudate cells were separated with a 9-ml Percoll gradient solution at room temperature in a 10-ml Beckman (Fullerton, CA) ultracentrifuge tube, and then the mixture was centrifuged for 20 min at 60,650 × g at 4°C. Neutrophils distributed in the second opaque layer were washed once with 10 ml of 5% FCS/RPMI 1640 medium, stained with Liu’s staining solution, and observed under conventional light microscopy to evaluate their purity. Cell viability was determined by an eosin Y exclusion assay. Usually, the neutrophil preparation obtained had a purity of >90% and a viability of 99%. CD11b, an activation marker of neutrophils, was stained by an Ab specific for mouse CD11b (M1/70, PE conjugated).

**Direct coculture of tumor cells with neutrophils and Transwell experiments**

Freshly isolated neutrophils or those fixed with 1% parafomaldehyde for 1 h at 4°C and tumor cells were cocultured in RPMI 1640–10% FCS at 37°C in a humidified CO2 incubator (5% CO2/95% air) for 20 h in six-well tissue culture plates (20, 34). To avoid cell-to-cell contact, neutrophils were separated from B16F10 transfectants by a 0.4-μm membrane in a well of Transwell six-well plates (Costar, Cambridge, MA). Neutrophils were placed in the upper chambers of the Transwell culture inserts. After 20 h, tumor cells from the lower chambers were harvested, washed, and analyzed for apoptosis, as described above. In some experiments, the interaction of Fas and FasL was interrupted by a 1-h preincubation using 500 ng mFasFc protein.

**SDS-PAGE gelatin zymography**

Release of gelatinase B was analyzed to indicate neutrophil activation. Tumor cells and neutrophils were cocultured for 20 h. Supernatants were collected. The gelatinase activity was determined by zymography, as described previously (35). Briefly, supernatants were subjected to electrophoresis on 7.5% SDS acrylamide gels containing 0.1% gelatin under nonreducing conditions. After electrophoresis, gels were stained by a solution containing 5% gelatin. After washing twice with sterile PBS, the gels were dehydrated, embedded in OCT compound (Miles, Elkhart, IN), frozen in liquid nitrogen, and stored at −20°C. Five-micrometer-thick cryosections were placed on poly(t-L-lysine)-coated glass slides and fixed with 3.7% formaldehyde in either PBS for 15 min plus acetone for 3 min or 3.7% parafomaldehyde for 5 min plus acetone for 1 min. The endogenous peroxidase activity in tissue sections was depleted by incubation in PBS containing 5% H2O2 for 30 min. The primary Ab was diluted with Ab diluent (DAKO, Carpenter CA), and included rat anti-NK mAb (DX5), rat anti-CD4 mAb (H12.19.2), rat anti-CD8 mAb (53-6.7), rat anti-Ly-6G (RB6-8C5) mAb, or rabbit anti-FasL (N-20). The secondary Abs were sheep anti-rat IgG conjugate or goat anti-rabbit IgG peroxidase. Redish-brown peroxidase staining was developed with an aminochel carbazole substrate kit. Sections were counterstained with hematoxylin and mounted in glycerol gelatin.

**Assay for reactive oxygen species (ROS) detection**

The oxidative burst of neutrophils can be monitored quantitatively using H2DCFDA. Intracellular H2DCFDA, a nonfluorescent fluorescein analog, can be oxidized to highly fluorescent DCF by activated neutrophils (36). Briefly, after 20 h of coincubation with tumor cells, neutrophils were harvested and incubated with serum-free medium containing 10 μM H2DCFDA at 37°C for 20 min. Fluorescence was monitored by FACScan with excitation at 488 nm and emission at 530 nm. This generation of ROS was completely suppressed by the addition of the antioxidant acetylcysteine.
but not that of FasLhigh tumors were palpable or FasLlow (Rn) tumor cells in the left flank, and tumor nodules FasLhigh tumor cells (V1, V2, V5, or V13) measured on days 12, 15, R5, R6, or R7) formed smaller tumors in mice as compared with cells, but not significantly affected by CD4/H11001 of FasLhigh tumors (V3, V4, or V5) in mice depleted of CD4/H11001 enhanced in neutrophil-depleted mice (Fig. 2 A). Neutrophil depletion enhanced the growth of FasLlow tumors, although FasL high tumors had a greater amount of neutrophilic infiltration, to our surprise, the development of FasLhigh tumors was not significantly enhanced by neutrophil-depleted mice (Fig. 2A). The average size of FastLhigh tumors (V3, V4, or V5) in mice depleted of CD4+/T cells was similar to that in mice that received control IgG on day 18 postinoculation. CD8+/T cells, or neutrophils by specific Abs (Fig. 2). Although FasLhigh tumors had a greater amount of neutrophilic infiltration, to our surprise, the development of FasLhigh tumors was not significantly enhanced by neutrophil-depleted mice (Fig. 2A). The average size of FastLhigh tumors (V3, V4, or V5) in mice depleted of CD4+/T cells was similar to that in mice that received control IgG on day 18 postinoculation. CD8+/T cell depletion did not affect the tumor size of V4 and V5, but reduced the size of V5. The size of V13 tumors was reduced by combined depletion of CD4+/CD8-/T cells, but not significantly affected by CD4+/neutrophils and CD8-/neutrophil cell depletion (Fig. 2B). In contrast, depletion of immune cells affected the size of FastLlow tumors to various extents. The average tumor volume of R4 was increased by CD4+/ or CD8+/T cell depletion. The size of R6 tumors in mice was significantly enhanced by CD8-/T cell depletion, but not by CD4+/T cell depletion. With neutrophil depletion, the size of FastLlow tumors (R4 and R6) increased by 2- to 4-fold on day 18 postinoculation (Fig. 2A). Multiple depletions of CD4+/CD8+/T cells, CD4+/neutrophils, and CD8+/neutrophils further enhanced the size of R4 (Fig. 2B).

**Results**

**FastLhigh tumor cells formed larger tumors with massive neutrophil infiltration than did FastLlow tumor cells**

C57BL/6 mice were given an injection of 5 × 10⁵ FastLhigh (Vn) or FastLlow (Rn) tumor cells in the left flank, and tumor nodules were palpable~10 days after inoculation. FastLlow tumor cells (R4, R5, R6, or R7) formed smaller tumors in mice as compared with FastLhigh tumor cells (V1, V2, V5, or V13) measured on days 12, 15, or 18 postinoculation (Table I). The average incidence of tumor formation was much lower in mice inoculated with FastLlow tumor cells (44% by day 12; 61% by day 15) than with FastLhigh tumor cells (100% by day 12; 100% by day 15).

Results of immunohistochemical staining showed reduced expression of FasL in tumors formed by FastLlow cells as compared with that by FastLhigh tumor cells (Fig. 1, A and D). Endothelial cells of blood vessels were also FasL positive (Fig. 1D). FastLhigh tumor cells showed more intensive neutrophilic infiltration accompanied by multiple necrotizing areas than did FastLlow tumor cells. A larger number of neutrophils were detected in FastLhigh tumors (Fig. 1, B and C) than in FastLlow tumors (Fig. 1, E and F). However, CD4+/T cells, CD8+ T cells, or NK cells were barely seen in tumors, regardless the levels of tumor FasL (data not shown).

**Neutrophil depletion enhanced the growth of FastLlow tumors, but not that of FastLhigh tumors**

To determine which immune cells were required for controlling tumor formation, mice were depleted of CD4+/T cells, CD8+ T cells, or neutrophils by specific Abs (Fig. 2). Although FastLhigh tumors had a greater amount of neutrophilic infiltration, to our surprise, the development of FastLhigh tumors was not significantly enhanced in neutrophil-depleted mice (Fig. 2A). The average size of FastLhigh tumors (V3, V4, or V5) in mice depleted of CD4+/T cells was similar to that in mice that received control IgG on day 18 postinoculation. CD8+ T cell depletion did not affect the tumor size of V3 and V4, but reduced the size of V5. The size of V13 tumors was reduced by combined depletion of CD4+/CD8-/T cells, but not significantly affected by CD4+/neutrophils and CD8-/neutrophil cell depletion (Fig. 2B). In contrast, depletion of immune cells affected the size of FastLlow tumors to various extents. The average tumor volume of R4 was increased by CD4+ or CD8+ T cell depletion. The size of R6 tumors in mice was significantly enhanced by CD8+ T cell depletion, but not by CD4+ T cell depletion. With neutrophil depletion, the size of FastLlow tumors (R4 and R6) increased by 2- to 4-fold on day 18 postinoculation (Fig. 2A). Multiple depletions of CD4+/CD8+ T cells, CD4+/neutrophils, and CD8+/neutrophils further enhanced the size of R4 (Fig. 2B).

**FastL conferred resistance in tumor cells to the cytotoxic activity of neutrophils in vitro**

We further investigated whether neutrophils could directly lyse tumor cells in vitro. Apoptotic cells were stained with PI and appeared as a sub-G₁/G₀ population in flow cytometric analysis. Murine neutrophils killed tumor cells at an E:T ratio of above 25:1 under coculture conditions (Fig. 3A). FastLhigh tumor cells were

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### Table I. Tumor volumes of FastLhigh and FastLlow derivatives in C57BL/6 mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>Day 12</th>
<th>Day 15</th>
<th>Day 18</th>
</tr>
</thead>
<tbody>
<tr>
<td>V1</td>
<td>280 ± 120 (5/5)</td>
<td>902 ± 224 (5/5)</td>
<td>1853 ± 411 (5/5)</td>
</tr>
<tr>
<td>V2</td>
<td>89 ± 65 (9/9)</td>
<td>662 ± 232 (9/9)</td>
<td>1648 ± 675 (9/9)</td>
</tr>
<tr>
<td>V5</td>
<td>437 ± 144 (3/3)</td>
<td>932 ± 277 (3/3)</td>
<td>1677 ± 454 (3/3)</td>
</tr>
<tr>
<td>V13</td>
<td>209 ± 22 (5/5)</td>
<td>674 ± 169 (5/5)</td>
<td>1097 ± 272 (3/3)</td>
</tr>
<tr>
<td>Average incidence</td>
<td>22/22 (100%)</td>
<td>22/22 (100%)</td>
<td>20/20 (100%)</td>
</tr>
<tr>
<td>R4</td>
<td>BD̅ (0/6)</td>
<td>6 (1/6)</td>
<td>140 ± 139 (6/6)</td>
</tr>
<tr>
<td>R5</td>
<td>108 ± 51 (3/3)</td>
<td>303 ± 97 (3/3)</td>
<td>774 ± 258 (3/3)</td>
</tr>
<tr>
<td>R6</td>
<td>8 (1/4)</td>
<td>77 ± 37 (2/4)</td>
<td>87 ± 53 (4/4)</td>
</tr>
<tr>
<td>R7</td>
<td>155 ± 42 (4/5)</td>
<td>227 ± 129 (5/5)</td>
<td>611 ± 433 (5/5)</td>
</tr>
<tr>
<td>Average incidence</td>
<td>8/18 (44%)</td>
<td>11/18 (61%)</td>
<td>18/18 (100%)</td>
</tr>
</tbody>
</table>

* a The fraction number in parentheses indicates tumor incidence.

* b Below detection.

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**Statistical analysis**

Results are expressed as the mean ± SE. Values were compared using Student’s t test for independent experiments. For multiple factors comparison between different groups, data were analyzed by a multway ANOVA (FasL, LPS, host, and time) using SAS statistical software package version 8.2 (SAS Institute, Cary, NC). Statistical significance was set at p ≤ 0.05.
more resistant to neutrophil-mediated apoptosis than were FasL\textsuperscript{low} tumor cells (compare V13 with R7 in Fig. 3A; V8, V11, and V13 with R4, R6, and R7 in Table II). In accordance with the results of PI staining, the TUNEL assay detected a higher percentage of apoptotic cells in FasL\textsuperscript{low} tumor cells cultured with neutrophils than in FasL\textsuperscript{high} tumor cells \((p < 0.001, \text{Fig. 3B})\). These results suggest that tumor FasL may inhibit neutrophil-mediated apoptosis in tumor cells.

To clarify whether neutrophil-mediated apoptosis requires cell-to-cell contact, tumor cells were cultured in cell supernatants, or with neutrophils either together or separately in a Transwell unit. Moreover, we also cultured tumor cells with paraformaldehyde-fixed neutrophils. As summarized in Table II, paraformaldehyde-fixed neutrophils were less cytotoxic to tumor cells. The supernatants obtained from cultures of tumor cells alone or from cocultures of neutrophils with tumor cells showed no cytotoxic activity to tumor cells. In addition, no cell killing was observed in the culture setting in which neutrophils and tumor cells were separated by a permeable membrane in a Transwell unit, further suggesting a cell-to-cell contact-dependent killing process. In contrast, contact with tumor cells prolonged the survival of neutrophils in vitro, which was irrelevant to the levels of FasL on tumor cells (Table II).

\textbf{Tumor FasL impaired the activation of neutrophils}

The above results showed that FasL\textsuperscript{high} tumor cells were more resistant to neutrophil-mediated apoptosis than were FasL\textsuperscript{low} tumor cells in vitro. To elucidate how tumor FasL affected the function of neutrophils, we analyzed gelatinase B release, ROS production, and CD11b expression of neutrophils. Neutrophils coincubated with FasL\textsuperscript{low} tumor cells (R4, R6, or R7) released more gelatinase B to the culture supernatants than did those with FasL\textsuperscript{high} tumor cells (V7, V8, or V13) \((p < 0.001, \text{Fig. 4A and C})\). In contrast, tumor cells did not release gelatinase B (Fig. 4B), indicating that the gelatinase activities detected in the culture medium were mainly produced by neutrophils. In coculture with FasL\textsuperscript{low} tumor cells (R4 or R7), the expression of CD11b on the surface of neutrophils was up-regulated compared with those with...
CD11b expression, neutrophils cocultured with FasLlow tumor cells with the suppressive effect of tumor FasL on gelatinase B release and produced more ROS than did those with FasLhigh tumor cells (V5, V11, or V13). We further used Student’s t test was used to compare FasL high and FasLlow tumors (p < 0.005; **, p < 0.001).

FasLhigh tumor cells (V8 or V13) (p < 0.001, Fig. 4D). In accordance with the suppressive effect of tumor FasL on gelatinase B release and CD11b expression, neutrophils cocultured with FasLlow tumor cells produced more ROS than did those with FasLhigh tumor cells (compare V8/V11 with R4/R7; p < 0.001, Fig. 4E). Neutrophils expressed Fas and FasL (Fig. 4F). Levels of Fas in neutrophils did not change under the culture conditions used in this study. Neutrophils cultured in vitro for 20 h expressed low level of FasL (Fig. 4F, 20 h). Activation of neutrophils by LPS or PMA enhanced the expression of FasL. Interestingly, the expression of FasL in neutrophils was significantly augmented by a 20-h coculture with FasLhigh tumor cells (R4 or R7), but not with FasLhigh tumor cells (V5, V11, or V13). We further used recombinant mFasFc chimera proteins to interfere with the interaction of Fas and FasL. Treatment with mFasFc did not significantly affect the spontaneous apoptosis of tumor cells cultured alone in vitro. In addition, adding mFasFc to the culture medium did not alter the basal ROS production of casein-enriched neutrophils. The ROS production of neutrophils upon contact with FasLhigh tumor cells (V11 or V13) was enhanced when tumor cells were pretreated with mFasFc for 1 h before coculture (Fig. 5A). In contrast, mFasFc did not affect the ROS production of neutrophils cocultured with FasLlow tumor cells. Accordingly, mFasFc pretreatment elevated the susceptibility of FasLhigh (V11 or V13) tumor cells to neutrophil killing, but not that of FasLlow cells (R4 or R7) (Fig. 5B). These results indicate thus that tumor FasL can impair the activation and oxidative bursts in neutrophils.

The antitumor activity of neutrophils in vivo

As the functions of neutrophils were impaired upon contact with FasLhigh tumor cells, we tested whether activating neutrophils by LPS inhibits tumor growth in vivo. An LPS injection slightly inhibited the growth of FasLhigh tumors (V5 or V13), while it significantly inhibited that of FasLlow tumors (R6 or R7) (Fig. 6). By day 12, the tumor incidence of C57BL/6 mice implanted with FasLlow tumor cells was lower in LPS-treated groups (4 of 8; average incidence, 50%) than in saline-treated groups (6 of 8; average incidence, 75%) (Fig. 6A). LPS can activate both neutrophils and B cells (37). To answer the question of whether LPS, in the absence of an adaptive immune response, is still capable of activating neutrophils to kill tumors, we grew tumors in NOD/SCID mice. Then LPS was serially injected into the tumor area. The melanoma transfectants showed the same tumorigenicity in NOD/SCID mice as those in C57BL/6 mice in terms of tumor size and tumor incidence. FasLlow tumors developed in NOD/SCID mice were more sensitive to LPS treatment by significant reduction in tumor volumes (Fig. 6, B and D). With an LPS injection, the incidence of tumor formation in NOD/SCID mice decreased (average incidence, 66.7% for FasLhigh tumors and average incidence, 0% for FasLlow tumors in LPS-treated groups compared with average incidence, 100% for FasLhigh tumors and average incidence, 62.5% for FasLlow tumors in saline-treated groups) (Fig. 6B).

As activating neutrophils by LPS delayed tumor formation, we further applied adoptive transfer of activated neutrophils to control tumor growth. To do this, casein-elicited or PMA-treated neutrophils were instilled into the tumor area. When locally transferred into the tumor mass, both PMA-activated neutrophils and casein-elicited neutrophils were effective in suppressing the growth of tumors (V13 and R4) (Table III). In addition, the reduction in

### Table II. Apoptosis in B16F10 derivatives and neutrophils in vitro

<table>
<thead>
<tr>
<th>Exp. I</th>
<th>Medium</th>
<th>Coculturea</th>
<th>Fixation</th>
<th>Cell Supernatants</th>
<th>Transwell</th>
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<tr>
<td></td>
<td></td>
<td>Percentage of apoptotic tumor cells (mean ± SE)</td>
<td></td>
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<tr>
<td>V8</td>
<td>0.57 ± 0.42</td>
<td>13.75 ± 1.78</td>
<td>4.50 ± 1.70</td>
<td>1.13 ± 0.73</td>
<td>1.13 ± 0.42</td>
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<tr>
<td>R4</td>
<td>1.10 ± 0.90</td>
<td>28.27 ± 2.51</td>
<td>12.10 ± 0.60</td>
<td>4.50 ± 0.20</td>
<td>7.03 ± 1.11</td>
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<tr>
<td>R7</td>
<td>0.70 ± 0.20</td>
<td>16.85 ± 0.55</td>
<td>6.43 ± 1.51</td>
<td>0.95 ± 0.55</td>
<td>1.05 ± 0.25</td>
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<tr>
<td>Fresh</td>
<td>2.74 ± 0.71</td>
<td>8.05 ± 1.40</td>
<td>10.15 ± 5.58</td>
<td>1.50 ± 0.75</td>
<td>2.70 ± 0.20</td>
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</table>

<table>
<thead>
<tr>
<th>Exp. II</th>
<th>Percentage of apoptotic tumor cells (mean ± SE)</th>
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<tbody>
<tr>
<td></td>
<td>V11 0.75 ± 0.35</td>
<td>10.85 ± 2.05</td>
<td>4.10 ± 0.40</td>
<td>3.10 ± 1.50</td>
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<tr>
<td></td>
<td>V13 1.08 ± 0.26</td>
<td>11.15 ± 1.25</td>
<td>2.95 ± 0.75</td>
<td>1.65 ± 0.45</td>
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<td></td>
<td>R6 0.78 ± 0.09</td>
<td>28.70 ± 4.50</td>
<td>5.45 ± 0.95</td>
<td>4.05 ± 0.35</td>
</tr>
</tbody>
</table>

*a Neutrophils were cocultured with tumor cells, as indicated, for 20 h.
average size of tumors by adoptive transfer of neutrophils was more profound for R4 than for V13.

Discussion

By using ribozymes to down-regulate the FasL gene, we found that FasL<sup>high</sup> melanoma cells, even though showing a slightly higher apoptotic rate in vitro (27) and causing neutrophil infiltration, developed larger tumors in C57BL/6 mice than did FasL<sup>low</sup> cells. Our results differ from those deduced from FasL overexpression systems, in which FasL caused tissue destruction due to inflammatory responses initiated by the recruitment of neutrophils (17–22). A possible explanation for this discrepancy is that the action of FasL in overexpression systems is too strong and thus initiates severe inflammation leading to tissue destruction. It was reported that the threshold level of FasL determines its biological consequences in vivo (28). A high level of membrane FasL on tumors is required to initiate detrimental inflammation, both in immune-privileged tissues and nonimmune-privileged sites such as the eye and the skin (28). We previously also found that the degree of local innate immunity in nude mice against human glioma cells was correlated with the levels of FasL (38). We speculate that only when the FasL gene is under the control of an intrinsic promoter does the amount of FasL on tumor cells reach a level sufficient to induce immune suppression in the environment of s.c. tissue. This also leads to the opinion that approaches with a down-regulation system may best reflect the reality for the function of the FasL gene in physiological conditions. It is noteworthy that the tumor-promoting effect of FasL on melanomas was observed only in s.c. tissues. By using these B16F10 transfectants, we found that a high level of FasL on melanoma cells retarded their metastatic ability in the lung of C57BL/6 mice (27). A study using an ocular tumor model showed that TGF-β1 within the aqueous humor of the eye suppresses the immune response, which is a major mechanism to maintain the immune privilege in the eye (28). Moreover, one Ag can trigger different immune responses, either Th1 or Th2, depending on where and how it is delivered into a host (39). Different lymphocyte subsets are present in bronchoalveolar lavage fluid and peripheral blood in healthy person (40, 41). Specifically, the ratio of helper-suppressor T cells is higher in the lung as compared with the peripheral blood. The FasL-sensitive lymphocytes will be selectively recruited or killed in the lung, as suggested by a responder trap model for immune tolerance in the liver (42). These results concur to support the concept that the local tissue environment may also determine the action of tumor FasL.

B16F10 transfectants formed tumors in NOD/SCID mice as quickly as in immune-intact C57BL/6 mice. We barely detected T, B, and NK cells in tumor regions. Unexpectedly, FasL<sup>low</sup> tumors grew faster in CD4<sup>+</sup> T cell-, CD8<sup>+</sup> T cell-, or neutrophil-depleted mice, although those cells did not intensively infiltrate into tumor sites as detected by immunohistochemical staining. In agreement with the established inflammation-inducing effect of FasL, down-regulation of FasL of melanoma cells reduced neutrophil infiltration. Interestingly, although FasL<sup>high</sup> tumors showed heavy neutrophilic infiltration, the size of tumor nodules progressively increased and eventually killed the mice. Moreover, neither depletion of neutrophils nor depletion of CD4<sup>+</sup> T cells significantly affected the development of FasL<sup>high</sup> tumors, indicating a FasL-associated suppressive mechanism operated in situ during the encounter between the tumor and immune cells. That CD8 depletion reduced the size of V5 tumor is a surprise finding. Similarly, double depletion of CD4<sup>+</sup> and CD8<sup>+</sup> cells reduced the size of V13. Previous studies showed that T cells could help the formation of
some tumors (43–45). A distinct profile of cytokines produced by those tumor-infiltrating T cells is believed to cause immune dysfunction and thus stimulate tumor formation (46, 47). However, this supposition cannot be applied as a general rule to all FasL<sup>high</sup> clones. Not all tumor clones were affected by CD8/CD4 depletion, suggesting that other unknown factor contributed to the tumor surveillance mechanism in host animals.

Results of the in vitro cytotoxicity assay showed that tumor cells became more susceptible to neutrophils when their FasL was down-regulated by ribozymes. In addition, the findings that paraformaldehyde-fixed neutrophils were less cytotoxic to tumor cells, that the addition of supernatants from coculture of tumor cells plus neutrophils failed to induce tumor cell apoptosis, and that the separation of neutrophils and tumor cells by the Transwell culture system prevented apoptosis in tumor cells indicate that tumor cell apoptosis induced by neutrophils involves surface proteins and requires cell-to-cell contact. We found no evidence that FasL<sup>high</sup> tumor cells could directly kill neutrophils. As a matter of fact, tumor cells reduced the spontaneous apoptosis in neutrophils cultured with tumor cells regardless of their levels of tumor FasL. Recently, we found that when in contact with human glioma cells, the spontaneous apoptosis in peripheral circulating neutrophils was reduced due to the elevated levels of IL-6 and IL-8 in culture medium. Furthermore, glioma cells produce these survival factors upon the activation of Fas (48). We believe that the prolonged survival of the murine neutrophils upon incubation with melanoma cells is operated through a similar mechanism by human neutrophils. Thus, direct cell cytotoxicity to deplete neutrophils cannot explain why FasL<sup>high</sup> tumor cells were less sensitive to neutrophils and suggests a functional deterioration in neutrophils induced by tumor FasL. To test this hypothesis, we examined the activities of neutrophils in detail, focusing on the productions of secretary proteases and ROS, which are major forces in tumor destruction (49–

**FIGURE 5.** Blockage of Fas and FasL interaction by mFasFc. Tumor cells were preincubated with mFasFc protein for 1 h and then were subjected to coculture with neutrophils (E:T ratio, 50:1) for 20 h. A, ROS production in neutrophils and B, tumor cell apoptosis detected by PI staining. Neutrophils cultured alone for 20 h served as a control. Values analyzed by Student's t-test differed between FasL<sup>high</sup> and FasL<sup>low</sup> tumor cells, which either received treatment with mFasFc protein or did not (*, p < 0.005; **, p < 0.001).

**FIGURE 6.** Tumor volumes of FasL<sup>high</sup> and FasL<sup>low</sup> derivatives in LPS-treated mice. FasL<sup>low</sup> or FasL<sup>high</sup> tumor cells at 5 x 10<sup>5</sup> were injected into the left flank of mice. A multiple-way ANOVA analysis on factors of FasL, LPS, host, and time compared tumor size on days 12 and 18 to determine the LPS effect (*, p < 0.05; **, p < 0.001).
51. We observed that the resistance of FasL<sup>high</sup> tumor cells to killing by neutrophils was correlated with impaired neutrophil activation, as demonstrated by reductions in gelatinase B secretion, ROS production, and the surface expression of CD11b. Neutrophils express Fas and FasL. Activating neutrophils by LPS, PMA, or coculture with FasL<sup>low</sup> tumor cells can enhance the expression of FasL in neutrophils. Interestingly, coculture with FasL<sup>high</sup> tumor cells did not augment the expression of FasL in neutrophils. As Fas/FasL system is one of the major cytotoxic mechanisms used by neutrophils, the loss of FasL on neutrophils upon cell contact may contribute to the resistance of FasL<sup>high</sup> tumor cells against neutrophil killing. Moreover, the application of mFasFc to block tumor FasL resulted in a greater amount of ROS production against neutrophil killing. Moreover, the application of mFasFc to block tumor FasL resulted in a greater amount of ROS production.

To test whether the restoration of neutrophil activity could inhibit tumor formation, we applied LPS and PMA to stimulate neutrophils. The tumor formation of FasL<sup>low</sup> transfectants in C57BL/6 mice was delayed by serial injections of LPS or by adoptive transfer of casein-sensitized or PMA-treated neutrophils. Similarly, in NOD/SCID mice, injection of LPS was also effective in slowing down the growth of tumors with low FasL, suggesting that adaptive immunity is not essential for LPS-induced neutrophil activation in antitumor immunity against FasL<sup>low</sup> tumors. However, FasL<sup>high</sup> tumor cells showed different responses to LPS treatment. Injection of LPS alone did not significantly affect the growth of FasL<sup>high</sup> tumors in either NOD/SCID or C57BL/6 mice. But when the neutrophils were activated in vitro and reInjected into tumor-bearing mice, they were effective in delaying the growth of FasL<sup>high</sup> tumors. These results suggest that tumor FasL causes local suppression of neutrophil activities very quickly, and the treatment with LPS alone cannot fully reactivate those neutrophils in tumors. Instead, preactivated neutrophils, which have not been affected by tumor FasL before cell contact, can suppress tumor growth in vivo. The pivotal role of innate immune cells in tumor combat has previously been recognized in several tumors, but combined therapy with drugs and LPS to improve innate immunity has exhibited various degrees of effectiveness against tumors (52–60). In this study, our results show that tumor FasL determines the effectiveness of innate immunity. Cell therapy with neutrophils, particularly those preactivated in vitro, may achieve a better antitumor effect than LPS treatment for tumors expressing high levels of FasL.

In conclusion, we demonstrate in this work that the expression of FasL on melanoma cells leads to neutrophil inactivation and helps tumor development in s.c. regions. An understanding of how tumor cells defeat neutrophils through the Fas/FasL pathway will facilitate the development of effective strategies that will be beneficial to antitumor therapy.

### References


