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Induction of Antitumor Immunity with Fas/APO-1 Ligand (CD95L)-Transfected Neuroblastoma Neuro-2a Cells

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Fas/Apo-1 (CD95)-Fas ligand (FasL) system has been implicated in the suppression and stimulation of immune responses. We examined the induction of antitumor immunity with neuroblastoma Neuro-2a cells transfected with FasL cDNA (Neuro-2a+FasL). Neuro-2a+FasL cells expressed FasL on the cell surface and secreted soluble FasL. Histologic and flow cytometric analyses revealed that Neuro-2a+FasL cells caused neutrophils to infiltrate into the injected site, resulting in strong inflammation. Neutrophil infiltration was inhibited by treatment with anti-FasL mAb and did not occur in Fas-deficient lpr mice. Normal syngeneic mice injected Neuro-2a+FasL cells after the inflammation and acquired tumor-specific protective immunity. CD8+ T cells were responsible for the antitumor immunity. Neuro-2a+FasL cells formed tumors after far longer latency compared with mock-transfected Neuro-2a+Neo cells in nude mice, and immune competent mice injected Neuro-2a cells but not sarcoma S713a cells when they were injected with Neuro-2a+FasL cells in a mixture. These results suggest that neutrophils attracted through the Fas-FasL system may impair tumor cells by inflammation at the initial step, followed by development of CD8+ T cell-dependent tumor-specific antitumor immunity, leading to complete eradication of tumor cells. Importantly, the treatment with Neuro-2a+FasL cells exhibited therapeutic efficacy against growing tumors. The Journal of Immunology, 1999, 162: 7350–7357.

The Fas/Apo-1 (CD95) receptor/ligand system induces apoptosis and plays an important role in the immune system (1–5). Fas is related to members of the TNF/nerve growth factor receptor family (6), and Fas ligand (FasL) is a member of the TNF/nerve growth factor family (3). Fas and FasL are broadly distributed on lymphoid and nonlymphoid cells (3, 6). FasL is also expressed at the sites of immune privilege such as testis (7), eye (8, 9), and placenta (10). Immunoaugmented tissues constitutively express FasL, and infiltrating Fas+ T cells and Fas+ granulocytes rapidly undergo FasL-induced apoptosis. Thus, the tissue is protected from immune-mediated damage. Similarly, allograft rejection of pancreatic islets is prevented with engineered myolasts expressing FasL (11). Overexpression of FasL in mouse ankle joints ameliorated collagen-induced arthritis, providing evidence for an antiinflammatory role of FasL in vivo (12). However, using the same strategy to abrogate the immune response to islets cells, allograft rejection was not always prevented by overexpression of FasL in the transplantation (13–15). A number of tumors including nonlymphoid tumors have been observed to express constitutively functional FasL (16–29). These tumor cells are capable of killing Fas+ Jurkat lymphocytes in vitro. Thereby, FasL-positive tumors may impair the tumor immune surveillance. In contrast, tumors expressing FasL can induce granulocyte-mediated tumor rejection (30, 31). Therefore, these reports are inconsistent with regard to the role of FasL in the regulation of the immune response and cause much controversy.

Neuroblastoma is the most common childhood malignant solid tumor arising from the sympathetic nervous system and characterized by a diversity of clinical behavior, ranging from spontaneous remission to rapid tumor progression (32). Thus, murine neuroblastoma Neuro-2a was selected as a model of human neuroblastoma and studied for the development of effective immunotherapy. We have studied the cytotoxic potential of soluble recombinant FasL in Fas+ lymphoma cells (Yac-1) implanted i.p. into mice (33). Culture supernatant of neuroblastoma Neuro-2a cells transfected with murine FasL cDNA (Neuro-2a+Neo) contained FasL and transduced a potent apoptotic signal to Fas+ Yac-1 cells. The soluble FasL was a full-length molecule (40 kDa) and not a proteolytically cleaved form. However, the effect of FasL on Yac-1 cells was assessed within 1 day after FasL injection. In the current study, we demonstrate that tumor cells expressing FasL, Neuro-2a+FasL, induced potent antitumor immunity associated with interference of tumor growth.

Materials and Methods

Mice

A/J (H-2b, syngeneic to Neuro-2a tumor cells), C3H/HeJ (C3H) (H-2b), C3H/HeJ-lpr/lpr (C3H-lpr) and BALB/c nu/nu (H-2d) mice were purchased from SLC (Hamamatsu, Japan). Double mutant mice, C3H-gld/gld lpr/lpr (C3H-gld/lpr), were constructed from C3H-gld and C3H-lpr and maintained in Laboratory Animal Research Center, Institute of Medical Science, University of Tokyo (34).
Tumors

FasL cDNA was obtained from C57BL mice. Neuro-2a (35), mock (neomycin-resistant cDNA)-transfected Neuro-2a (Neuro-2a+Neo), and Neuro-2a+Fasl. cells (33) were maintained in DMEM (Iwaki, Tokyo) containing 10% heat-inactivated FBS, 4.5 g/L glucose, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Rockville, MD) at 37°C under 5% CO₂. A Neuro-2a tumor mass was aseptically removed 3 wk after i.d. injection into AJ mice and gently dissociated in PBS with a cell strainer (Falcon, Becton Dickinson, Mountain View, CA). After washing with PBS, tumor cells were cultured and used for the in vivo implantation. F6b (Neo+ Fas⁺) and N1d (Neo⁺) cells were produced from murine hepatoma MHI34 cells (Fas⁺) (34). These three tumor cell lines were passaged by culture in RPMI 1640 medium containing 10% FBS, 2-ME (5 × 10⁻⁵ M), and kanamycin. S713a sarcoma cells were maintained in RPMI 1640 medium containing 5% FBS, penicillin, and streptomycin.

Cytotoxicity test

Cell-free culture supernatants were collected after about 6 × 10⁶ Neuro-2a+Fasl. and Neuro-2a+Neo cells were incubated in 20 ml of DMEM containing 1% FBS for 2–3 days. F6b or N1d cells suspended in 100 μl of culture medium were incubated with each of the supernatants for 18–20 h in a 96-well microtiter plate. Anti-Fasl. mAb (clone K10, mouse IgG2b) (36) was harvested as ascites from SCID mice and purified with ammonium sulfate precipitation. For anti-Fasl. mAb treatment, the supernatant (36) was harvested as ascites from SCID mice and purified with ammonium sulfate precipitation. Mice were immunized by i.d. injection of 10⁶ Neuro-2a or S713a cells (5 × 10⁵) on day 0. Neuro-2a+Fasl. and Neuro-2a cells were injected at different sites; either rostral and caudal sites about 2 cm distant from each other in the right flank or right and left flank, respectively. For investigation of the influence of Fasl.-expressing cells on tumor formation, Neuro-2a+Fasl. (5 × 10⁶) were mixed with Neuro-2a (5 × 10⁵) or S713a (5 × 10⁵) tumor cells and injected i.d. into AJ mice. In a therapeutic experiment, AJ mice injected i.d. with 5 × 10⁶ Neuro-2a or S713a cells on day 0 were treated by injecting viable Neuro-2a+Fasl. or MMC-treated Neuro-2a (10⁶) cells on days -1 and 0 of the tumor cells. Anti-Fasl. mAb treatment was significantly active against Neuro-2a tumor cells as early as 3 days after injection. Anti-Fasl. mAb (K10) and control IgG2b were used as isotype control.

In vivo antitumor activity

Neuro-2a cells were incubated with mitomycin C (MMC) (60 μg/ml) at 37°C for 40 min to deteriorate in vivo transplantability. In an immunoprophylactic experiment, AJ mice were injected i.d. with viable Neuro-2a+Fasl., MMC-treated Neuro-2a+Fasl., or MMC-treated Neuro-2a (10⁶) cells on day -1 and challenged i.d. with Neuro-2a or S713a cells (5 × 10⁶) on day 0. Neuro-2a+Fasl. and Neuro-2a cells were injected at different sites; either rostral and caudal sites about 2 cm distant from each other in the right flank or right and left flank, respectively. For investigation of the influence of Fasl.-expressing cells on tumor formation, Neuro-2a+Fasl. (5 × 10⁶) were mixed with Neuro-2a (5 × 10⁵) or S713a (5 × 10⁵) tumor cells and injected i.d. into AJ mice. In a therapeutic experiment, AJ mice injected i.d. with 5 × 10⁶ Neuro-2a or S713a cells on day 0 were treated by injecting viable Neuro-2a+Fasl. or MMC-treated Neuro-2a (10⁶) cells on days 0 and 3. Anti-Fasl. mAb treatment was significantly active against Neuro-2a tumor cells as early as 3 days after injection. Anti-Fasl. mAb (K10) and control IgG2b were used as isotype control.

In vivo inactivation of CD4⁺ and CD8⁺ T cells with anti-CD4 and anti-CD8 mAbs

Anti-CD4 (JK1.5) and anti-CD8 (2.43) mAbs were harvested as ascites from nude mice. Anti-CD4 mAb was used after purification with ammonium sulfate precipitation. Mouse were immunized by i.d. injection of 10⁶ Neuro-2a+Fasl. cells on day -14, i.p. administered with 0.5 mg of purified anti-CD4 mAb on days -3 and -1 to deplete CD4⁺ T cells or 0.2 ml of ascitic anti-CD8 mAb on days -3 and 0 to deplete CD8⁺ T cells, and i.d. injected with 5 × 10⁵ Neuro-2a cells on day 0. CD4⁺ T cells were reduced from 18% to 0.4% and CD8⁺ T cells from 10.5% to 0.1% in the spleen of these mice.

Flow cytometry of cells infiltrated with Neuro-2a+Fasl.

Mice were i.d. injected with 10⁶ Neuro-2a+Fasl. cells, a small but palpable tissue mass which formed at the injected site was excised 24 h later, and single-cell suspensions were prepared by teasing tissue masses with a cell strainer (Falcon) in PBS containing 0.6 mM EDTA. After washing with PBS, cells were incubated with anti-CD16/CD32 (FcγRIIIA) (PharMin- gen, San Diego, CA) to block FcR-mediated binding of Ab and then stained with FITC-anti-Gr-1 (Ly-G6) (rat IgG2b) (Caltag, South San Francisco, CA) (37), FITC-anti-Mac-1 (CD11b) (rat IgG2b) (Caltag) (38), FITC-anti-CD8 (Becton Dickinson), and/or biotin-anti-Fas Jo2 (hamster IgG) mAbs (PharMingen) and PE-streapavidin (Life Technologies), PE-anti-CD4 mAb (Becton Dickinson), and analyzed with a FACSCalibur (Becton Dickinson). Biotin-hammer IgM and FITC-rat IgG2b (Caltag) were used as isotype control.

Histology

A/J, C3H, C3H-lpr, and C3H-gld/lpr mice were i.d. injected with 10⁶ Neuro-2a+Fasl., Neuro-2a+Neo, or Neuro-2a cells at the age of 6–8 wk. Some A/J mice were injected i.p. with 125 μg of anti-Fasl. (K10) 8 h and 30 min before the transplantation of Neuro-2a+Fasl. cells. The skin at the injected site was examined for the presence or absence of swelling and then cut out 24 h and 3 and 5 days later. The skin samples were fixed in 10% formalin in PBS, processed routinely, embedded in paraffin, sectioned at 3 μm, and stained with hematoxylin and eosin for microscopy.

Results

Characterization of Fasl. expressing in Neuro-2a+Fasl. cells

Culture supernatants from Neuro-2a+Fasl. and Neuro-2a+Neo cells were incubated with the hepatoma cells Fasl⁺ F6b and Fasl⁺ N1d cells to examine the induction of apoptosis by Fasl. (Fig. 1A). Supernatant from Neuro-2a+Fasl. but not Neuro-2a+Neo cells

FIGURE 1. Characterization of Neuro-2a+Fasl. cells. A. Supernatants from Neuro-2a+Fasl. and Neuro-2a+Neo cells were incubated with F6b and N1d cells. Supernatant from Neuro-2a+Fasl. cells against F6b cells (●), supernatant from Neuro-2a+Neo cells against F6b cells (○), supernatant from Neuro-2a+Fasl. cells against N1d cells (▲), and supernatant from Neuro-2a+Neo cells against N1d cells (△). B. Supernatant from Neuro-2a+Fasl. cells was pretreated with anti-Fasl. mAb and control IgG2b, followed by the addition of F6b cells. The original concentrations of mAb and control IgG2b were 20 mg/ml. The results of one of more than three similar experiments are presented. Bar and asterisk indicate the SD of the mean and statistical significance (*, p < 0.0001; †, p < 0.0001; ‡, p < 0.0001; and §, p < 0.0001) by Student’s t test vs tumor control, respectively.

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induced the cytotoxicity against F6b cells. The cytotoxic activity of supernatant from Neuro-2a+FasL cells against F6b cells was abrogated by treatment with anti-FasL mAb and not with control mouse IgG2b (Fig. 2), indicating that the effector molecule in the supernatant was FasL. In the flow cytometry, Neuro-2a+FasL cells were stained with anti-FasL K10 but not with control mouse IgG2b (Fig. 2). These results evidenced that Neuro-2a+FasL cells secreted soluble FasL into culture medium and expressed FasL on the surface. Fas and FasL were not detected in Neuro-2a cells. Neuro-2a+FasL cells did not express Fas and were resistant to FasL-mediated killing (data not shown).

Loss of tumorigenic potential of Neuro-2a cells by FasL transfection

To investigate the functional consequences of FasL expressed by Neuro-2a cells, syngeneic A/J mice were injected with 10⁶ Neuro-2a+FasL cells after formation of a small but clearly palpable tumor mass on days 1–4 (Table I). When 10⁶ Neuro-2a+FasL cells were injected into A/J (n = 19) and C3H (n = 10) mice, a clearly swelled mass as huge as 6 × 6 to 8 × 8 mm in size appeared at the injected site 24 h later. This mass formation was inhibited by pretreatment with injection of anti-FasL mAb K10 (<1 × 1 mm) in A/J mice (n = 7) and occurred in neither C3H-lpr (<1 × 1 mm) (n = 3) nor C3H-gld/lpr (<1 × 1 mm) (n = 6) mice, which carry a functional defect in Fas alone and both FasL and Fas (34), respectively, indicating that the Fas-FasL system is directly involved in this event. In contrast, tumor growth occurred without such instant mass formation accompanied in all the mice injected with 5 × 10⁵ Neuro-2a+FasL or parent Neuro-2a cells (Fig. 3 and Table I).

Histology

As mentioned above, a palpable swelling developed immediately at the site where Neuro-2a+FasL cells were injected. Thus, 10⁶ tumor cells were injected and the injected sites were examined histologically. At 24 h later, abundant leukocytes, mainly neutrophils, infiltrated into and around tumor cells in A/J and C3H mice that were injected with Neuro-2a+FasL cells and underwent swelling (Fig. 4A). Some neutrophils appeared to be in contact with tumor cells. On day 5 of Neuro-2a+FasL cell injection, dead tumor cells and their debris were surrounded by granulomatous tissues in which many neutrophils were scattered (data not shown). In contrast, such swelling did not occur and only a few neutrophils and monocytes were found around tumor cells in A/J mice injected with Neuro-2a+FasL (data not shown). More importantly, Neuro-2a+FasL cells induced neither swelling nor leukocyte infiltration in C3H-lpr (data not shown) and C3H-gld/lpr (Fig. 4C) mice. Thus, Fas played a pivotal role in induction of an inflammatory response by FasL-transfected tumor cells. Similar massive accumulation of inflammatory cells consisting of neutrophils has been reported for other FasL-transfected tumor cells (30, 31).

Flow cytometry of cells infiltrating around Neuro-2a+FasL cells

To further characterize the cells responsible for the swelling, free cells were isolated from a tissue mass formed 24 h after injection of 10⁶ Neuro-2a+FasL cells and analyzed by flow cytometry. The isolated cells were smaller than Neuro-2a+FasL cells in size (Fig. 5). The forward and side scatter histogram of the infiltrated cells consisted of a high peak of the smallest cells such as erythrocytes and cell debris (forward scatter (FSC) = 22, side scatter (SSC) = 21) and a low peak of lymphoid-like cells (FSC = 73, SSC = 41). The histogram of Neuro-2a+FasL cells showed a major peak of tumor cells (FSC = 105, SSC = 85), a minor peak of cell debris (FSC = 19, SSC = 20), and a bottom (FSC = 60, SSC = 25). Thus, flow cytometry was performed by gating the low peak to exclude the smallest cells and tumor cells. As shown in Fig. 6, A and B, almost all of these cells (>90%) were stained with anti-Gr-1 (Ly-6G) mAb specific for granulocytes (37) and anti-Mac-1
Induction and tumor specificity of protective immunity

<table>
<thead>
<tr>
<th>Immunization</th>
<th>Challenge</th>
<th>Tumor Incidence (%)</th>
<th>Tumor Size (mm)</th>
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<tbody>
<tr>
<td>Neuro-2a+FasL (right flank)</td>
<td>Neuro-2a (right flank)</td>
<td>2/19 (11)*</td>
<td>17.9 (n = 2), 0 (n = 17)*</td>
</tr>
<tr>
<td>Neuro-2a+FasL (right flank)</td>
<td>Neuro-2a (left flank)</td>
<td>1/7 (14)†</td>
<td>17.3 (n = 1), 0 (n = 6)§</td>
</tr>
<tr>
<td>MMC-Neuro-2a+FasL</td>
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<td>5/7 (71)</td>
<td>15.4 ± 3.7 (n = 5), 0 (n = 2)¶</td>
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<td>MMC-Neuro-2a</td>
<td>Neuro-2a</td>
<td>5/5 (100)</td>
<td>17.5 ± 3.6</td>
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<tr>
<td>PBS</td>
<td>Neuro-2a+Neo</td>
<td>5/5 (100)</td>
<td>17.0 ± 1.4</td>
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<tr>
<td>PBS</td>
<td>Neuro-2a</td>
<td>13/13 (100)</td>
<td>20.2 ± 2.2</td>
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<tr>
<td>Neuro-2a+FasL</td>
<td>S713a</td>
<td>8/8 (100)</td>
<td>18.2 ± 2.9</td>
</tr>
<tr>
<td>PBS</td>
<td>S713a</td>
<td>8/8 (100)</td>
<td>19.1 ± 2.8</td>
</tr>
</tbody>
</table>

* p < 0.001 by Fischer’s exact probability test vs PBS + Neuro-2a control.
† p < 0.0001 by Mann-Whitney test vs PBS + Neuro-2a control.
‡ p < 0.0004 by Fischer’s exact probability test vs PBS + Neuro-2a control.
§ p < 0.001 by Mann-Whitney test vs PBS + Neuro-2a control.
¶ p < 0.001 by Mann-Whitney test vs PBS + Neuro-2a control.

Incomplete eradication of Neuro-2a+FasL cells in nude mice

To determine whether or not T cells are required for eradication of Neuro-2a+FasL cells, the tumor cells were implanted into nude mice. All nude mice suppressed the growth of Neuro-2a+FasL cells but not of Neuro-2a+Neo cells as shown in Fig. 9. However, five of six nude mice injected with Neuro-2a+FasL cells developed tumors after a latent period as long as 3 wk. Thus, it became clear that T cells are essential for complete rejection of Neuro-2a+FasL cells in addition to neutrophils.

Establishment of protective antitumor immunity after eradication of Neuro-2a+FasL cells

Neuro-2a+FasL cells were eradicated spontaneously and Neuro-2a cells were also eradicated when injected together with Neuro-2a+FasL cells. Hereupon, to address whether or not protective immunity was established after rejection of Neuro-2a+FasL cells, mice were challenged with Neuro-2a cells 14 days after injection of Neuro-2a+FasL cells (Table I). They manifested a strong immunoprotective effect (rejection rate: 17 of 19, 89%) for >60 days after inoculation of Neuro-2a cells. In contrast, immunization with MMC-treated Neuro-2a or MMC-treated Neuro-2a+Neo cells (data not shown) or PBS alone did not induce antitumor immunity. Moreover, mice were immunized with MMC-treated Neuro-2a+FasL cells as a control (Table I). The antitumor

(CD11b) mAb specific for granulocytes, macrophages, and NK cells (38), indicating that the infiltrating cells were granulocytes consistent with the histological findings (Fig. 4). Importantly, about 20% of the cells were positive for anti-Fas Ab, albeit the possibility that Fas on the cells might be occupied with FasL. About 20% of the cells stained with anti-Fas Ab compared with anti-CD4 Ab. Moreover, the possibility that Fas on the cells might be occupied with FasL. Approximately 140.

FIGURE 4. Histology of the sites where 10⁶ tumor cells were injected 24 h previously. Large cells on the top are the injected tumor cells. A, Abundant leukocytes, mostly neutrophils, infiltrate between and around Neuro-2a+FasL cells in an A/J mouse. Neutrophils surround tumor cells and appear to be in contact with tumor cells. Note the larger area infiltrated and far higher density of leukocytes compared with B and C. The same result was also observed in C3H mice. B, Small numbers of neutrophils and monocytes infiltrate around Neuro-2a+Neo cells in an A/J mouse. C, No neutrophils are found around Neuro-2a+FasL cells in a C3H-gld/lpr mouse. Original magnification, ×140.

CD11b mAb specific for granulocytes, macrophages, and NK cells (38), indicating that the infiltrating cells were granulocytes consistent with the histological findings (Fig. 4). Importantly, about 20% of the cells were positive for anti-Fas Ab, albeit the possibility that Fas on the cells might be occupied with FasL. Approximately 140.
immunity induced was significantly less potent in these mice than in those injected with viable Neuro-2a+FasL cells. MMC-treated Neuro-2a+FasL cells neither released FasL in vitro nor developed palpable swelling in vivo. These results indicated that the active production of FasL was necessary for the induction of antitumor immunity. When challenge with Neuro-2a cells was given at the site either ipsilateral or contralateral to the immunized site, the same degree of antitumor immunity was observed (Table I), indicating that Neuro-2a+FasL cells can induce systemic immunity. Importantly, S713a cells formed tumors at the same growth rate in both immunized and control mice in support of the induction of tumor-specific antitumor immunity with Neuro-2a+FasL cells (Table I).

Role of CD4+ and CD8+ T cells in protective antitumor immunity

To address whether CD4+ or CD8+ T cells were responsible for tumor-specific antitumor immunity, the immunized mice were injected with anti-CD4 or anti-CD8 mAb before challenging with Neuro-2a cells (Table II). Flow cytometric analysis indicated that Neuro-2a cells were CD4+CD8− (data not shown). Anti-CD8 but not anti-CD4 mAb treatment completely abrogated antitumor immunity, demonstrating that CD8+ T cells were required for the rejection of tumor cells in protective immunity.

Therapeutic effect of Neuro-2a+FasL cells

As Neuro-2a+FasL cells conferred strong antitumor immunity on mice, we addressed the possibility of their application to tumor therapy. Mice were injected with Neuro-2a cells, followed by...
immunization with Neuro-2a.

Table II. Characterization of antitumor effector T cells induced by immunization with Neuro-2a+FasL cells

<table>
<thead>
<tr>
<th>Immunization</th>
<th>mAb Treatment</th>
<th>Tumor Incidence (%)</th>
<th>Tumor Size (mm)</th>
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<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>Anti-CD4</td>
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<td>0*</td>
</tr>
<tr>
<td>+</td>
<td>Anti-CD8</td>
<td>8/8 (100)</td>
<td>19.0 ± 4.8</td>
</tr>
<tr>
<td>-</td>
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</tr>
<tr>
<td>-</td>
<td>-</td>
<td>7/7 (100)</td>
<td>18.2 ± 1.8</td>
</tr>
</tbody>
</table>

* Two weeks after i.d. injection with Neuro-2a+FasL, (10⁶) cells, mice were i.d. challenged with Neuro-2a (5 × 10⁶). Some mice were treated with anti-CD4 and anti-CD8 mAb to deplete CD4⁺ and CD8⁺ T cells, respectively, before challenge. Tumor incidence and tumor size were determined at 3 wk after the challenge.

† p < 0.0004; and † p < 0.002 by Fisher’s exact probability test vs Neuro-2a control.

‡ p < 0.0001 by Mann-Whitney test vs Neuro-2a control.

Discussion

In this study, we showed that in contrast to FasL-negative tumors, tumor cells that express FasL on cell surface and release FasL were rejected in a syngeneic system (Figs. 1, 2, 3, and 10 and Table I). Neuro-2a+FasL cells were found to induce protective and therapeutic immunity against parent Neuro-2a cells (Table I and Fig. 10). Thus, FasL produced locally acted as an immunostimulatory rather than an immunosuppressive cytokine, and promoted potent antitumor immunity. FasL produced by melanoma cells has been shown to abrogate the antitumor immune response (16). The findings presented here show the opposite, namely FasL-mediated enhancement of tumor immune surveillance. Of note is that Neuro-2a cells transfected with FasL cDNA produce FasL in membrane form (mFasL), which is the bioactive form in contrast to the cleaved soluble peptide. In contrast, Kayagaki et al. (36) reported polymorphism of murine Fasl with different biological activity. We showed that less potent FasL that was derived from C57BL mice induced remarkable antitumor immunity (Table I and Fig. 10). However, immunological privilege was also proved using less potent FasL (7, 9, 11). Thus, the two opposite phenomena, immunostimulation and immunological privilege (immunosuppression), may not be explained by polymorphism in Fasl.

Seino et al. (30) and Arai et al. (31) reported that FasL induced granulocyte-mediated inflammation, leading to tumor rejection. Ligation of Fas can induce secretion of IL-8 (39), which might contribute to the ensuing of inflammation. Histological examination and flow cytometric analysis revealed the prompt infiltration of neutrophils into the i.d. site where Neuro-2a+FasL cells were injected (Figs. 4 and 6). These neutrophils partly expressed Fas on the surface. In addition, infiltration of neutrophils into the tumor site was not observed in Fas-deficient mice. These results suggested that the host response resulting in tumor rejection may be initiated by Fas-Fasl system-mediated recruitment of neutrophils to the tumor.

To elucidate the antitumor mechanism of FasL-producing tumor cells, S713a sarcoma cells mixed with Neuro-2a+FasL cells were inoculated into mice (Fig. 8). Neuro-2a+FasL cells did not inhibit the growth of S713a tumors despite the fact that they exhibited strong antitumor activity against parent Neuro-2a cells. Direct killing of S713a by FasL did not occur because S713a is a Fas⁻ tumor. These results were very important for elucidating the role of neutrophils in antitumor response. Neuro-2a+FasL cells release Fasl, which is known to have chemotactic activity to neutrophils (40). The neutrophils that were massively accumulated in the tumor site were in close contact with tumor cells (Fig. 4A). Neutrophils may release various cytokines including proinflammatory cytokines (IL-1, IL-8, TNF) (41) and damage tumor cells (42, 43). If the neutrophils exert their antitumor activity indirectly through the cytokines, bystander tumor S713a tumor cells may also undergo damage. However, Neuro-2a+FasL cells did not affect S713a cells at all (Fig. 8). Thus, the direct contact between neutrophils and tumor may be necessary for neutrophils to attack tumor cells. Neutrophils displayed enzyme- and nitric oxide-mediated lytic activity against tumor cells (43, 44). In contrast, neutrophils discriminated between G-CSF-producing and G-CSF-nonproducing tumor cells and repressed only the former cells by direct contact with them.
Neuro-2a+FasL cells formed tumors after a far longer latent period compared with Neuro-2a+Neo cells in nude mice (Fig. 9), indicating that neutrophils cannot eradicate tumor cells completely without T cells. In support of this, immune competent A/J mice rejected Neuro-2a+FasL cells inoculated alone (Fig. 3), Neuro-2a cells injected as a mixture with Neuro-2a+FasL cells (Fig. 7) and even growing Neuro-2a cells after treatment with Neuro-2a+FasL cells (Fig. 10). As a result, they acquired tumor-specific immunological memory, because they could not reject S713a sarcoma cells (Table I). CD8+ T cells were responsible for the established rejection of tumor-bearing mice (Table II). In addition, neutrophils were required to induce antitumor effector CD8+ T cells in mice (45) and rats (46). These results suggest that neutrophils alone can inhibit growth of Neuro-2a+FasL cells at least temporarily and that CD8+ T cells are essential for the complete rejection of Neuro-2a+FasL cells.

Neuro-2a+FasL cells not only express FasL on the cell surface but also release the bioactive soluble full-length FasL (Figs. 1 and 2) (33). We have recently reported that i.v. but not i.p. injection of Neuro-2a+FasL-derived FasL induced apoptosis of liver cells with liver failure within 2 h of injection (33). However, we confirmed that i.d. injection of Neuro-2a+FasL (105) cells caused neither clinically detrimental effects such as loss of body weight nor impairment of liver function as determined by serum glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase, and total bilirubin levels 24 h after the injection in wild-type mice (data not shown). Thus, toxicity of Neuro-2a+FasL cells may require significant levels of mFasL in the serum, while local expression of mFasL at the injection site or in different tissues may be relatively harmless.

The genetic modification of tumor cells for the local expression of cytokines IL-1, IL-2, IL-4, IL-12, IFN-γ, and TNF has recently been studied as a novel approach to the active immunotherapy of tumors. Neuro-2a cancer (47). Immunization with the modified tumor cells promoted regression of tumor cells. This pathway may be amplified by direct FasL-mediated tumor cell death. A further study is needed to elucidate the FasL-mediated antitumor mechanism.

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REFERENCES


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