Cutting Edge: A Novel Role for Fas Ligand in Facilitating Antigen Acquisition by Dendritic Cells

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Fas ligand (FasL) is a type II integral membrane protein of the TNF family and mediates apoptosis of a variety of cell types including activated T cells (1). Although earlier studies indicated that FasL might contribute to immune evasion by inducing apoptosis of activated T cells (2), recent studies revealed that forced expression of FasL in tumors triggers a potent neutrophil-mediated local inflammatory response and targets the FasL-bearing tumors for rejection (3,7). Moreover, FasL-bearing tumor cells were rejected not only in immunocompetent mice, but also in T cell-deficient nude mice (4), suggesting that FasL-triggered primary rejection per se is T cell independent. Following this primary rejection, a T cell-dependent, tumor-specific protective immunity was also established in immunocompetent mice (4). To further investigate the mechanism of FasL-mediated systemic immunity, we established FasL transfectants of two histologically distinct tumor lines, A11 Lewis lung carcinoma (8) and B16 melanoma, and examined their tumorigenicity in vivo when mixed at various ratios with either their own parental cells or distinct tumor cells. We found that FasL induced a specific immune response against Ag derived from FasL-bearing tumors. In addition, we show that dendritic cells (DCs) efficiently interacted with FasL-expressing tumor cells and that DCs cocultured with FasL tumors were able to elicit a tumor-specific immune response in vivo. These findings reveal a new role for FasL on tumor cells in facilitating tumor-DC interaction and subsequent tumor Ag acquisition by DCs, and suggest potential usage of FasL-bearing tumor cells in the generation of tumor-specific DC-based vaccines.

**Materials and Methods**

**Mice and tumor cells**

Six- to 10-wk-old female C57BL/6 (B6), B6/lpr/lpr, and BALB/c nu/nu mice were purchased from Japan SLC (Shizuoka, Japan) and maintained in our animal facility under the specific pathogen-free conditions. A11 and P29 are high- and low-metastatic clones, respectively, isolated from Lewis lung carcinoma (8). B16 is a mouse melanoma cell line. All tumor cell lines are of B6 origin. Cells were cultured in DMEM containing 100 U/ml kanamycin supplemented with 10% FCS.

**Antibodies**

Biotinylated Abs (anti-Fas, Jo-2; anti-FasL, K10; FITC-conjugated Abs (B220, CD11b, Thy-1, CD40), PE-conjugated Abs (CD80 and CD86), and streptavidin-conjugated PE were purchased from BD PharMingen (San Diego, CA).

**DC culture**

Bone marrow cells were first depleted of erythrocytes by incubating in a buffer containing 0.15 M NH4Cl, 1 mM KHCO3, and 0.1 mM EDTA (pH 7.4) at 25°C for 5 min. After washing with PBS, T cells, B cells, and macrophages were depleted by incubating with a mixture of biotinylated Abs against CD4, CD8, B220, TER119, CD11b, FITC-conjugated Abs (B220, CD11b, Thy-1, CD40), PE-conjugated Abs (CD80 and CD86), and streptavidin-conjugated PE were purchased from BD PharMingen (San Diego, CA).

**Coculture of DCs with tumor cells**

DCs and tumor cells were labeled with PKH67 and PKH26, respectively, according to the manufacturer’s protocol (Sigma-Aldrich, St. Louis, MO). For DC-mediated antitumor experiments, 3 × 106 unlabeled DCs were cocultured for 12 h with 1 × 106 PKH26-labeled tumor cells and stained
with FITC-conjugated anti-Thy-1 Ab. Thy-1high, PKH26-negative cells were then isolated using a FACSVantage cell sorter. For DC-tumor interactions, 1 × 10⁶ PKH67-labeled day 10 DCs were cocultured for 12 h with 3.3 × 10⁷ PKH26-labeled tumor cells in 6-cm culture dishes and then analyzed under a fluorescence microscope (DMI20E; Leica Microsystems, Wetzlar, Germany).

Results
FasL-expressing tumor cells trigger T cell-dependent tumor-specific immunity and prevent the growth and metastasis of subsequently inoculated FasL negative tumors

We first examined in vivo tumor growth of A11 Lewis lung carcinoma and B16 melanoma and their FasL transfectants (A11/FasL and B16/FasL). A11/FasL and B16/FasL were promptly rejected in immunocompetent syngeneic B6 mice (Ref. 9 and data not shown). The rejection also occurred in T cell-deficient nude mice but not in Fas-defective B6/lpr mice (9), confirming that this primary rejection is T cell independent, but requires Fas expression by host cells (4). B6 mice that rejected A11/FasL were protected from lethal challenges of parental A11 cells and, to a lesser extent, a related carcinoma P29, but not B16 cells (Fig. 1A). In nude mice that rejected A11/FasL, parental A11 cells were able to grow at a similar growth rate as in naive nude mice (Fig. 1B), indicating that FasL-induced protective immunity requires T cells. A11 cells are highly metastatic and form pulmonary metastases when injected i.v. (8). Fig. 1C shows that s.c. inoculation of live A11/FasL cells 1 wk after the i.v. injection of A11 cells dramatically decreased the number of metastatic foci in the lung, indicating that FasL on tumor cells induced a systemic immunity.

FasL triggers tumor-specific protective immunity

To investigate the role of T cells during the primary response, we examined the ability of FasL transfectants to mediate the rejection of the coinoculated FasL negative cells in B6 and nude mice. A11/FasL effectively triggered the rejection of the parental A11 cells when they were mixed at ratios of 1:1 and 1:5 and coinjected into B6 mice (Fig. 1D). In contrast, A11/FasL mixed with A11 at a ratio of 1:5 were not rejected in nude mice (Fig. 1D). These observations suggest that the rejection of the parental A11 cells in B6 mice was not simply due to a bystander effect, but rather attributable to a T cell-dependent immune response. To further clarify the T cell dependency, we examined the Ag specificity of FasL-mediated primary rejection. We mixed A11/FasL with B16 melanoma, and examined tumor progression in B6 mice. The growth of B16 tumors was not compromised in mice coinjected with 10-fold more A11/FasL (Fig. 1E). Conversely, the growth of A11 cells was unaffected by the presence of 10-fold more B16/FasL (Fig. 1F). In both cases, A11/FasL and B16/FasL were readily rejected. These data clearly show that FasL-mediated primary rejection is tumor-specific, and suggest that tumor Ag-specific T cells participate in the primary antitumor response.

**FIGURE 1.** FasL-mediated primary rejection is tumor-specific. A. Induction of A11-specific immunity in mice that rejected A11/FasL. B6 mice were first inoculated s.c. with 2 × 10⁶ A11/FasL cells and 2 wk later challenged with 2 × 10⁵ parental A11 (●), an A11-related tumor line P29 (▲), or B16 melanoma (▲). B. Protective immunity is not evoked in nude mice. Nude mice that rejected 2 × 10⁶ A11/FasL (○) or naive nude mice (●) were s.c. inoculated with 2 × 10⁵ A11 cells. C. Suppression of pulmonary metastasis in the pre-existing tumors by FasL-transfectants. B6 mice were injected i.v. with 1 × 10⁶ A11 cells. One week later, mice were inoculated s.c. with PBS, 2 × 10⁴ A11 or A11/FasL cells. The lung metastases were enumerated 2 wk thereafter. D. Rejection of parental A11 cells by A11/FasL in B6 but not in nude mice. Parental A11 (●) or A11/FasL (▲) cells (2 × 10⁵), 2 × 10⁵ A11/FasL mixed with 2 × 10⁵ A11 cells (1:1, ○), or 4 × 10⁴ A11/FasL mixed with 2 × 10⁵ A11 cells (1:5, □) were implanted s.c. into B6 mice. A total of 4 × 10⁵ A11/FasL mixed with 2 × 10⁵ A11 cells (1:5, □) were also inoculated into nude mice (△). E and F, FasL transfectants did not trigger “a bystander rejection” of a distinct tumor line. A total of 2 × 10⁶ B16 (E, ▲), 2 × 10⁶ A11/FasL mixed with 2 × 10⁵ B16 cells (E, △), 2 × 10⁵ A11 (F, ○), or 2 × 10⁵ B16/FasL mixed with 2 × 10⁵ A11 cells (F, △) were inoculated s.c. into syngeneic B6 mice.
Efficient tumor-DC interactions mediated by membrane-bound FasL on tumor cells and Fas on DCs

The T cell-dependent specific immune response against FasL-bearing tumors implicates that putative tumor Ag have to be presented to T cells by APC. Therefore, we examined whether FasL expression on tumor cells augmented the tumor Ag acquisition by DCs, the most potent APC that are capable of sensitizing naive T cells and initiating primary immune responses (10, 11). When bone marrow-derived DCs were cocultured with A11/FasL, we observed efficient DC-tumor interactions as revealed by cluster formations (Fig. 2, B and C). In contrast, few clusters were formed between FasL negative parental tumor cells and DCs (Fig. 2A). To further confirm the direct contact between DCs and FasL-bearing tumor cells, we cocultured PKH67 (green)-labeled DCs with PKH26 (red)-labeled A11/FasL. DCs were found to be in close contact with tumor cells (Fig. 2, D and E, arrows). To clarify whether the DC-tumor interactions were dependent on the Fas/FasL system, we cocultured A11/FasL with DCs derived from Fas-defective lpr mice. We found little interactions occurred between these cells (A11/FasL + lpr/DC, Fig. 2F). Moreover, few clusters were observed after coculture of DCs with tumor cells expressing a soluble form FasL (A11/SolFasL, Fig. 2F). These results demonstrate that membrane-bound FasL on tumor cells and Fas on DCs mediate direct tumor-DC interactions.

DCs cocultured with FasL-bearing tumor cells elicit tumor-specific immunity in vivo

We next examined whether DCs cocultured with tumor cells were able to elicit a tumor-specific immune response. The bone marrow-derived DCs expressed Fas, CD40, CD80, CD11b, and MHC class II molecules on their cell surfaces and were composed of a Thy-1{high} and a Thy-1{dull} population (Fig. 3A and data not shown). Unlabeled DCs were cocultured with PKH26 (red)-labeled FasL-transfected or parental tumor cells (Fig. 3B) and then stained with FITC-conjugated Ab to Thy-1 (Fig. 3, C and D). The Thy-1{high} population was then purified with a FACS (gate R1; Fig. 3, C and D). We chose Thy-1 instead of other surface markers to purify DCs because the Thy-1{high} population could be easily separated from tumor cells with little chance of tumor contamination. Sorted DCs were mixed with FasL negative tumor cells and implanted s.c. into B6 mice. DCs cocultured with A11/FasL strongly prohibited tumor progression of the parental A11 cells, whereas DCs cocultured with B16/FasL or the parental A11 cells had little effect on the in vivo growth of A11 cells (Fig. 3E). Conversely, the growth of B16 tumors was inhibited only by DCs cocultured with B16/FasL, but not by DCs cocultured with A11/FasL (Fig. 3F). These data provide compelling evidence that during the coculture of DCs with tumor cells, DCs acquired tumor Ag from FasL-expressing cells and such Ag-loaded DCs effectively elicited tumor-specific immunity in vivo.

Discussion

FasL has been shown to induce apoptosis (1) and inflammation (4–7). The results of this study reveal a new role of FasL in mediating tumor-DC interaction and facilitating Ag acquisition by DCs, and suggest a unique approach in the generation of tumor-specific DC-based treatment. DCs cocultured with FasL-expressing tumors effectively prevented the growth in vivo of the parental cells, but not the growth of tumor cells of different origin. The absence of cross-protection indicates that DCs were loaded with putative tumor Ag during the coculture with FasL-expressing tumor cells. Because little interactions occurred between DCs and FasL-negative cells or cells expressing SolFasL or between DCs derived from Fas-defective lpr mice and FasL-positive tumor cells, DC-tumor contact and subsequent tumor Ag transfer to DCs are thus dependent on membrane-bound Fas and FasL.

The requirement for cell-cell contact to generate antitumor responses explains why FasL-expressing tumor cells could mediate the rejection of FasL negative autologous tumors without affecting the growth of distinct tumors. We speculate that DCs acquired Ag predominantly from FasL-expressing tumors and induced a specific immune response against these Ag. Consequently, the coincubated parental tumors that had the same antigenicity were rejected, while tumors of different origin, which likely expressed

FIGURE 2. Fas/FasL-mediated DC-tumor interaction. Parental A11 (A) or A11/FasL (B and C) cells were cocultured with DCs for 12 h. Arrows indicate DC-tumor clusters. C, A greater magnification of B (inset). D and E, PKH67 (green)-labeled DCs were cocultured with PKH26 (red)-labeled tumor cells. D, Phase contrast. E, Fluorescence micrograph. F, The number of DC-tumor clusters observed after coculture of DCs with tumors. The average number (with SE bars) of DC-tumor clusters in randomly selected 30 visual fields is shown.
different tumor Ag, were not affected. The lack of interaction between DCs and tumor cells expressing SolFasL (Fig. 2F) is consistent with the finding that soluble form FasL was unable to induce an antitumor immunity (Ref. 7 and data not shown). These observations indicate that direct cell-cell contact between DCs and tumors mediated by Fas/FasL interaction plays an essential role in the induction of tumor-specific immune responses.

Bone marrow-derived DCs express Fas on their cell surfaces but are resistant to Fas-mediated apoptosis (12). Recently, Rescigno et al. (13) showed that DCs underwent Fas-mediated maturation, implying that DCs may mature during their interactions with cognate T cells which are FasL positive. We have examined the expression of MHC class II and CD86 on DCs after the coculture with FasL transfectants or after the stimulation with CD40 ligand. Although CD40 ligand induced apparent up-regulation of both molecules, coculture with FasL transfectants did not significantly alter their expression (data not shown), suggesting that DCs were not fully activated after the coculture. It is possible that DCs cocultured with FasL transfectants undergo further maturation after in vivo administration.

DCs have recently been shown to be able to acquire Ag from apoptotic, necrotic, and whole-cell lysates (14, 15), and cross-present the Ag to induce tumor-specific cytotoxic T cells. In some cases, Ag-loaded DCs were shown to be effective vaccines to protect mice against a lethal challenge with tumor cells, although the efficacy differed depending on the maturation stage of DCs, the way of Ag loading and the administration route of DCs. In contrast, immature DCs that captured Ag from apoptotic cells may induce tolerance rather than immunity (16). Although the identification of an efficient Ag loading strategy remains a challenge, the results of the present study provide an alternative approach to load tumor Ag on DCs by using FasL-transfected live tumor cells. Interestingly, in contrast to live FasL+ tumors, UV-irradiated, apoptotic FasL+ cells were unable to induce a tumor-specific immunity when inoculated s.c. (data not shown), possibly because these apoptotic cells were quickly cleared in vivo by macrophages before they can interact with DCs.

A recent report indicated that DCs were able to capture Ag from live hemopoietic cells including B cells, T cells, macrophages, and DCs (17). The interaction between DCs and hemopoietic cells appears not to depend on Fas/FasL system because these cells do not normally express FasL. Hemopoietic cells may express certain adhesion molecules that facilitate their interactions with DCs and thus allow efficient Ag transfer to DCs. When unlabeled immature DCs were cocultured with fluorescence-labeled hemopoietic cells, immature DCs captured Ag from hemopoietic cells and became positive for the fluorescence. Similarly, PKH67 (green)-labeled DCs that captured PKH26 (red)-labeled apoptotic or necrotic cells could be easily identified by the presence of red fluorescence inside the “green” DCs under a fluorescent microscope, or by the appearance of a double-positive population in FACS analysis (17–21). In striking contrast with these previous findings, we have not observed apparent incorporation of labeled tumor cells or tumor-derived fragments into DCs (Fig. 2F), nor could we detect a double-positive population by FACS analysis (Fig. 3, C and D) after the coculture. Nevertheless, the direct contact observed between DCs and FasL-expressing tumor cells, along with the finding that such DCs were able to elicit a tumor type-specific immune response, strongly implicate that DCs acquired putative tumor Ag during the coculture. Further studies are required to reveal the precise mechanism by which tumor Ag are transferred to DCs. It remains to be investigated whether any receptor-ligand pair that bridges DC-tumor interaction would be able to induce a tumorspecific immunity.

In conclusion, we have found a novel function for FasL in facilitating tumor-DC interaction and subsequent tumor Ag acquisition by DCs. In addition, we show that DCs cocultured with FasL-expressing tumor cells are able to elicit tumor-specific immune responses. Although future studies are required to compare the efficacy and specificity of DC-based vaccines generated by different strategies, use of FasL to load tumor Ag on DCs could represent an alternative approach in cancer immunotherapy.

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References
confer immune privilege and instead targets them for rapid destruction. Nat. Med. 3:738.


