Vaccination of Dendritic Cells Loaded with Interleukin-12-Secreting Cancer Cells Augments In vivo Antitumor Immunity: Characteristics of Syngeneic and Allogeneic Antigen-Presenting Cell Cancer Hybrid Cells

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ABSTRACT
Cancer immunotherapy by fusion of antigen-presenting cells and tumor cells has been shown to induce potent antitumor immunity. In this study, we characterized syngeneic and allogeneic, murine macrophage/dendritic cell (DC)-cancer fusion cells for the antitumor effects. The results showed the superiority of allogeneic cells as fusion partners in both types of antigen-presenting cells in an in vivo immunotherapy model. A potent induction of tumor-specific CTLs was observed in these immunized conditions. In addition, the immunization with DC-cancer fusion cells was better than that with macrophage-cancer fusion cells. Both syngeneic and allogeneic DC-cancer fusion cells induced higher levels of IFN-γ production than macrophage-cancer fusion cells. Interestingly, allogeneic DC-cancer fusion cells were superior in that they efficiently induced Th1-type cytokines but not the Th2-type cytokines interleukin (IL)-10 and IL-4, whereas syngeneic DC-cancer fusion cells were powerful inducers of both Th1 and Th2 cytokines. These results suggest that allogeneic DCs are suitable as fusion cells in cancer immunotherapy. To further enhance the antitumor immunity in the clinical setting, we prepared DCs fused with IL-12 gene-transferred cancer cells and thus generated IL-12-secreting DC-cancer fusion cells. Immunization with these gene-modified DC-cancer fusion cells was able to elicit a markedly enhanced antitumor effect in the in vivo therapeutic model. This novel IL-12-producing fusion cell vaccine might be one promising intervention for future cancer immunotherapy.

INTRODUCTION
Cancer cells evade host immune surveillance at least in part due to their low immunogenicity (1). Reduced or absent expression of MHC class I (2), TAP-1 and TAP-2 (3, 4), or costimulatory molecules (5, 6) have been reported in malignant tumors. Strategies to augment the host immune response against cancer cells include vaccinations by gene-modified tumor cells or pulsing antigen-presenting cells (APC) with tumor-associated antigens (7). Recent studies have shown that vaccination of tumor cells fused with APCs such as dendritic cells (DC) or B cells could effectively elicit a host T-cell–mediated antitumor response (8–10). Such fusion cells present tumor-specific antigens, including known and unknown molecules, together with costimulatory molecules. Furthermore, fusion cells of allogeneic DCs and tumor cells express allogeneic MHC molecules, which would also stimulate alloreactive T cells (11–14) and support the induction of a potent reaction against tumor cells. These advantages of allogeneicity prompted us to investigate whether the allogeneic APC-cancer fusion cells induce a stronger reaction than syngeneic fusion cells.

Interleukin (IL)-12 is a heterodimeric proinflammatory cytokine produced by macrophages, DCs, and B cells, which stimulates T lymphocytes and natural killer cells to release IFN-γ and promotes an antitumor response (15, 16). Recent studies from us as well as others have shown that IL-12 has powerful adjuvant properties, and the immune-modulating characteristics of IL-12 are considered to be responsible for such adjuvant effects (17–20).

In this study, we first compared the antitumor effect of syngeneic and allogeneic APC-cancer fusion cells. Secondly, we investigated the adjuvant effect of secreted form of IL-12 on fusion cell immunotherapy. A combination of allogeneic APCs and IL-12-secreting cancer cells may provide us with a promising strategy for cancer therapy.

MATERIALS AND METHODS
Mice. C57BL/6 (H-2b) and BALB/c (H-2d) female mice, 6 to 8 weeks old, were obtained from Japan Charles River (Atsugi, Japan).
Cell Culture. The colon-26 adenocarcinoma cell line (C26) (H-2d) and BALB/3T3 fibroblast cell line (H-2b) were obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). The cell lines were maintained in complete RPMI 1640 (10% fetal bovine serum, 100 μg/mL streptomycin, and 100 units/mL penicillin). DCs were generated from mouse bone marrow precursors in complete RPMI 1640 with 10 ng/mL recombinant mouse granulocyte-macrophage colony-stimulating factor (R&D Systems, Minneapolis, MN) and 2 ng/mL recombinant mouse IL-4 (R&D Systems) as described previously (21). Briefly, on days 2 and 4, nonadherent cells were gently removed and fresh medium was added. On day 6, loosely adherent proliferating DC aggregates were dislodged and replated. On day 8 of culture, released nonadherent cells with the typical morphologic features of DCs were used for the experiment. Macrophages were harvested by peritoneal lavage with ice-cold PBS 4 days after i.p. injection with 2 mL sterile 4% thioglycollate broth. For phenotypic analysis, DCs and macrophages were stained with a panel of antibodies, including MHC class I and II, CD80, CD86, CD11b, and CD11c (PharMingen, San Diego, CA) and quantified by flow cytometry (Becton Dickinson, San Jose, CA).

Fusion Cells. The fusion cells were generated by electrical pulse (electrofusion; refs. 22, 23). Cells were mixed in 0.3 mol/L sucrose and first aligned by direct current at 100 V for 10 seconds. Electrofusion was driven by a Bio-Rad gene pulser (Bio-Rad, Hercules, CA; 600 V, 25mFD). DC and C26 were stained with CellTracker green CMFDA and orange CMTMR fluorescent dyes (Molecular Probes, Eugene, OR), respectively. DC, C26, mixture of DC and C26 (Mix), and DC-C26 fused cells (Fusion) were analyzed by flow cytometry. To verify surface phenotype of the fusion cells, C26 tumor cells were prelabeled with CMTMR. After electrofusion, both syngeneic and allogeneic DC-cancer fusion cells were detected by staining with FITC-conjugated antibodies against MHC class II, CD80, CD86, CD11b, and CD11c (PharMingen, San Diego, CA) and quantified by flow cytometry (Becton Dickinson, San Jose, CA).

Generation of IL-12-Secreting C26 Cells. The retroviral vector, TFG-m-IL-12 (kindly provided by Dr. H. Tahara, University of Tokyo, Tokyo, Japan), encodes both p35 and p40 murine IL-12 subunits with the IRES construct and selectable neomycin phosphotransferase gene under long terminal repeat control (24). C26 cells (5 × 10^5) were infected with the viral supernatant of the producer cell line (24) and subsequently selected by G418 (600 μg/mL). The fusion cells expressed both tumor and DC surface molecules, with a panel of antibodies, including MHC class I and II, CD80, CD86, CD11b, and CD11c (PharMingen, San Diego, CA) and quantified by flow cytometry (Becton Dickinson, San Jose, CA).

RESULTS

Generation of DC-Tumor Fusion Cells and Antitumor Effect. DCs were stained with CellTracker green CMFDA and cancer cells (C26) with orange CMTMR. Both cells were then subjected to electrofusion, which generated CMFDA-CMTMR double-positive fusion cells at consistent yield of approximately 30% (Fig. 1A). The fusion cells expressed both tumor and DC surface molecules, such as MHC class I (H-2Kd on C26 cancer cells and H-2Kb on C57BL/6 mice-derived allogeneic DC) and class II (I-Ab), CD80, CD86 (Fig. 1B), CD11c, and ICAM-1 (data not shown). Both
syngeneic and allogeneic DC-cancer fusion cells showed comparable phenotype (syngeneic DC-cancer fusion cells: MHC class II 23.7 ± 3.1%, CD80 33.9 ± 3.8%, CD86 28.8 ± 2.4%, CD11c 23.1 ± 1.5%, ICAM-I 23.9 ± 2.7%; allogeneic DC-cancer fusion cells: MHC class II 20.4 ± 4.8%, CD80 35.2 ± 4.5%, CD86 25.9 ± 2.9%, CD11c 21.9 ± 3.1%, ICAM-I 24.4 ± 4.5%). Mice treated with only irradiated cancer cells or with simple mixture of syngeneic DCs and cancer cells did not show significant antitumor effects compared with that with PBS control. Immunization with the syngeneic DC-cancer fusion cells significantly more suppressed the tumor volume than immunization with PBS or mixture of syngeneic DCs and cancer cells (Fig. 1C).

**Antitumor Effect by Syngeneic and Allogeneic APC-Cancer Fusion.** Examination of the syngeneic and allogeneic APCs as fusion partners revealed significant antitumor effect with the use of allogeneic APCs (Fig. 2). We compared the antitumor immunity elicited by syngeneic DC-cancer fusion cells and allogeneic DC-cancer fusion cells, where tumor inoculated mice were immunized on days 3 and 10 with syngeneic (BALB/c) DC-cancer fusion or allogeneic (C57BL/6) DC-cancer fusion cells. As shown in Fig. 2A, allogeneic DC-cancer fusion cells exerted a significantly more antitumor effect than the syngeneic DC-cancer fusion cells (tumor volume on day 19: PBS control 839 ± 69 mm³, syngeneic DC-cancer fusion 333 ± 81 mm³, and allogeneic DC-cancer fusion 215 ± 40 mm³). To confirm the induction of tumor-specific CTL, the splenocytes from mice immunized with both fusion cells were analyzed after restimulation with cancer cells. Splenocytes immunized by either syngeneic or allogeneic DC-cancer fusion cells showed tumor-specific cytotoxicity against C26 target cells (Fig. 2B). The cell lysis was not observed for BALB/3T3 fibroblast target cells (data not shown), indicating the lysis is C26 cancer cell specific.

We then examined the effects of allogeneic or syngeneic fusion cells with other APCs on tumor immunity. Peritoneal macrophages generated fusion cells at a similar yield with DCs (~30%), and the fusion cells expressed MHC class I and II, CD11b, CD80, and CD86 (data not shown). Mice treated with the macrophage-cancer fusion cells showed significant antitumor effects in the therapeutic model (tumor volume on day 19: PBS control 839 ± 69 mm³, syngeneic macrophage-cancer fusion 477 ± 95 mm³, and allogeneic macrophage-cancer fusion 316 ± 70 mm³; Fig. 2C). The allogeneic macrophage-cancer fusion cells showed stronger antitumor immunity than did the syngeneic macrophage-cancer fusion cells. Tumor-specific CTLs against C26 target cells were induced by syngeneic and allogeneic macrophage-cancer fusion cells (Fig. 2D), although nonspecific cell lysis against BALB/3T3 cells was not observed (data not shown).

**Functional Analysis of Splenocytes from Immunized Mice.** Analysis of the cytokine production of splenocytes from mice immunized with APC-cancer fusion cells revealed significant differences according to the DC-macrophage and the syngeneic or allogeneic background (Fig. 3). IFN-γ production from splenocytes was twice as high in mice immunized with DC-cancer fusion cells than in those immunized with macrophage-cancer fusion cells (Fig. 3A). The TNF-α production showed similar profiles in the mice with the DC-cancer combination and those with the macrophage-cancer combination (Fig. 3C). In marked contrast, both IL-4 and IL-10 production were high in splenocytes from mice immunized with...
syngeneic DC-cancer fusion cells but not in those with the other combinations (Fig. 3B and D). TGF-β production was not affected in the mice with the syngeneic or allogeneic and DC-macrophage combination (Fig. 3E). To characterize the cytokine-producing cells, we specifically analyzed IFN-γ and IL-4 secretion by purified CD4⁺ or CD8⁺ T cells. Whereas IFN-γ was secreted from mainly CD4⁺ and partly CD8⁺ T cells, IL-4 was produced only by CD4⁺ T cells but not by CD8⁺ T cells (Fig. 3A and B). The differences in these cytokine profiles might have affected the antitumor activity.

In vivo Functional Analysis of IL-12-Secreting DC-Tumor Fusion Cells. IL-12 is a pivotal cytokine that elicits strong antitumor effects by stimulating immune cells, including T cells and natural killer cells (15). To enhance the antitumor immunity by DC-cancer fusion cells, cancer cells that had been retrovirally transferred the secreted form of IL-12 gene were fused...
with syngeneic or allogeneic DCs (Fig. 4). The IL-12 transfected C26 cells expressed murine IL-12 (2,796 ± 122 pg/mL/10^6 cells/24 hours), and the derived fusion cells showed large amounts of IL-12 production (syngeneic DC-C26 fusion: 1,548 ± 265 pg/mL/10^6 cells/24 hours; allogeneic DC-IL-12 C26 fusion: 1,593 ± 289 pg/mL/10^6 cells/24 hours; Fig. 4A). The IL-12 production was not affected by 40 Gy irradiation before vaccination (data not shown). When tumor-bearing mice were immunized with IL-12-secreting fusion cells on days 3 and 10, augmented antitumor immunity was observed compared with the mice immunized with allogeneic DC-cancer fusion cells (tumor volume on day 19: PBS control 839 ± 69 mm³, syngeneic DC IL-12 fusion 169 ± 30 mm³, and allogeneic IL-12 DC fusion 157 ± 15 mm³; Fig. 4B). Although IL-12-secreting allogeneic DC-cancer fusion cells showed no stronger effect than IL-12-secreting syngeneic ones in vivo, tumor-specific CTL induction was more powerful in IL-12-secreting allogeneic DC-cancer fusion cells (Fig. 4C). The cytokine production by splenocytes immunized with IL-12-secreting fusion cells showed a similar profile to that by immunized with DC-cancer fusion cells without IL-12 secretion.

Fig. 3 Cytokine production from immunized splenocytes. Tumor-bearing mice were treated with syngeneic DC-C26 fusion, allogeneic DC-C26 fusion, syngeneic macrophage(Mφ)-C26 fusion, allogeneic Mφ-C26 fusion, or PBS control on 3 and 10, and the splenocytes harvested on day 20 were cocultured with mitomycin C–treated C26 cells. Secretion of (A) IFN-γ, (B) IL-4, (C) TNF-α, (D) IL-10, and (E) TGF-β from splenocytes was determined by ELISA (n = 5 in each group). Analysis of IFN-γ and IL-4 secretion by purified CD4^+ or CD8^+ T cells from immunized splenocytes were shown in A and B. Light gray bars, cytokine secretion by CD4^+ T cells; dark gray bars, CD8^+ T cells.
Whereas IFN-γ and TNF-α secretion were not affected by IL-12 gene transfer, both IL-4 and IL-10 secretion were significantly suppressed by IL-12 expression (Figs. 3C and D). Specific analysis showed that CD4+ T cells were involved in the secretion of IFN-γ and IL-4 and CD8+ T cells also have some role of producing IFN-γ (Fig. 4D). IL-12-secreting DC-cancer fusion cells most suppressed the tumor growth (Fig. 5).

**DISCUSSION**

Without destroying the immune barrier mechanisms of established solid tumors, there can be no effective therapy to eradicate cancer cells (25). Vaccination of tumor cells artificially fused with APCs is an attractive approach to overcome the immune escape mechanism that results from the reduced MHC expression or suppressed T-cell activation in the tumor-bearing condition (8–10, 22, 23, 26–34). The theoretical advantages of
immunogenic fusion cells include the possible involvement of both known and unidentified tumor antigens and further immune activation. Among several kinds of cancer immunotherapy, the use of synthetic short peptide antigens has been widely applied (7). A recent report, however, described the occurrence of post-translational protein splicing in a human renal cancer (32) and a melanoma cell (33), suggesting yet unknown immune escape mechanism. A method using whole tumor cells with APCs, including the tumor-DC fusion strategy, is possibly effective against such cancer cells.

In this study, we showed for the first time that macrophages as well as DCs are a promising fusion partner for generating a hybrid vaccine to be used in the treatment of cancer. Originally, B cells were used as a fusion partner for an effective tumor vaccine by Guo et al. (8). Gong et al. first used DCs as a fusion partner and reported that immunization with DC-cancer fusion cells induced the rejection of established metastasis in mice experiments (10). Several reports showed intriguing results with human DC-cancer fusion cells, demonstrating a regression of metastatic renal cell carcinoma (22), metastatic melanoma (26), and glioma (27). However, macrophages have never been proposed as a fusion partner. Because macrophages play an important role in the host defense against pathogens, wound healing, angiogenesis, various types of acute and chronic inflammation, and cancer immunity, the utilization of macrophages for fusion with cancer cells seemed worth investigating. Although we directly induced electrofusion after harvesting thioglycollate-stimulated peritoneal macrophages without any further in vitro treatment, the vaccination with these products showed effective antitumor activity in our therapeutic mouse model (Fig. 2C and D). Differences in the effects between DCs and macrophages might have been due to the lower production of IFN-γ shown in the macrophage-cancer cell fusion experiment (Fig. 3A). However, a possible advantage of the use of macrophages is the availability and abundance of such cells: human alveolar macrophages can be harvested by bronchoalveolar lavage and readily transformed into fusion cells, sterilized by irradiation, and vaccinated.

We next showed allogeneic APCs were better fusion partner with cancer cells than syngeneic APCs. Although several studies of cancer immunotherapy using fusion cells with allogeneic DCs and autologous tumor cells have been conducted in clinical settings (22), there has been little evidence supporting the validity of using allogeneic cells (28, 29). Recent reports on the existence of peripheral T cells responsive to alloantigen provide convincing support for the notion of using allogeneic DC-cancer fusion cells to induce a stronger reaction than that by syngeneic fusion cells. The frequency of peripheral T cells that recognize alloantigen has been reported to be between 1% and 10%, higher than the percentage of T cells that respond to foreign antigens (11-13), suggesting the advantage of allogeneicity as a vaccination compartment.

To clarify the underlying mechanism of the superior antitumor effect by allogeneic DC-cancer fusion cells, we examined the tumor-specific CTL activities and the cytokine profiles of splenocytes from immunized mice. Although there were no different CTL activities between mice treated with syngeneic and allogeneic DC-cancer fusion cells (Fig. 2B), several different cytokine profiles were detected between them (Fig. 3). Allogeneic DC-cancer fusion cells elicited a stronger antitumor effect because they induced sufficient secretion of Th1 cytokine IFN-γ and lower production of Th2 cytokines IL-4 and IL-10 than syngeneic DC-cancer fusion cells did (Figs. 3 and 4). In contrast, immunization with syngeneic DC-cancer fusion cells efficiently induced the production of both Th1- and Th2-type cytokines (Fig. 3). Further analysis revealed that CD4+ T cells mainly secreted IFN-γ, suggesting the important role of Th1 CD4+ T cells, and CD8+ T cells also contribute to produce IFN-γ (Fig. 3A). IL-4 was secreted only by CD4+ Th2 cells in mice treated with syngeneic DC-cancer fusion cells (Fig. 3B). Tanaka et al. (30) reported that IL-10 production induced by syngeneic DC-cancer fusion cells caused insufficient tumor eradication and that systemic IL-12 administration was required for effective antitumor effects in mice experiment. In contrast, we showed a significant therapeutic antitumor effect especially by allogeneic DC-cancer fusion cells, which could effectively elicit a cytokine profile of high IFN-γ and TNF-α and low IL-4 and IL-10 (tumor growth suppression compared with tumor volume of PBS control on day 19: syngeneic DC-cancer fusion 60% reduction and allogeneic DC-cancer fusion 74% reduction; Figs. 2A and 5). These findings showing allogeneic DC as a superior fusion partner are consistent with those of a recent report of on the use of allogeneic DCs by Siders et al. (29).

Although the method of fusing APCs and cancer cells can be used to suppress tumor growth, additional immunomodulation will be required to further potentiate the immune response. For

Fig. 5 Summary of antitumor effect by immunization with fusion cells. Average tumor volume on day 19 immunized with PBS control, syngeneic macrophage-Mφ-cancer fusion, allogeneic Mφ fusion, syngeneic DC fusion, allogeneic DC fusion, syngeneic DC IL-12 fusion, and allogeneic DC IL-12 fusion cells were plotted (n = 10 in each group).
this purpose, we investigated the use of IL-12 gene transfer to cancer cells and examined the effect of IL-12 secretion in the local milieu by gene-modified fusion cells. Immunization with both syngeneic and allogeneic IL-12 gene-modified fusion cells showed highly augmented antitumor effects in our therapeutic model (tumor growth suppression compared with tumor volume of PBS control on day 19: syngeneic DC-IL-12-cancer fusion 76% reduction and allogeneic DC-IL-12-cancer fusion 78% reduction; Figs. 4B and 5). In addition, DC-IL-12-cancer fusion cells induced stronger tumor-specific CTL activities compared with DC-cancer fusion cells (no gene-modified fusion cells; Figs. 2B and 4C). Furthermore, IL-12 gene-modified fusion cells elicited strong Th1 response and enhanced the suppression of the Th2 response (Figs. 3 and 4D), thus inducing augmented antitumor immunity (Fig. 4B). Although DC-IL-12-cancer fusion cells strategy led to the good results, no difference was observed between antitumor effects by using syngeneic and allogeneic DCs as fusion partners. This is partly because that the enough effect by IL-12 canceled out the advantage of allogeneicity. Studies on fusion cells composed of DCs and gene-modified cancer cells have provided further insight in tumor immunology. Kao et al. (34) reported that mice vaccinated with TGF-β-secreting fusion cells had lower tumor-specific CTL activity and had lower survival after tumor challenge as compared with control animals. Liu et al. (35) showed that IL-4 gene-modified fusion vaccine enhanced antitumor immunity in a tumor protection model, suggesting the possible importance of stimulating the DC maturation mechanism. In contrast, the present study showed that IL-12-secreting fusion cells directly enhanced tumor-specific CTL activity, induced stronger Th1 but suppressed Th2 cytokine production, and exerted potent antitumor effects in vivo. Among the variety of the choices of immune modulatory molecules used in preclinical animal models, the IL-12 gene-modified vaccine through affecting the accumulating host immune cells shows promise for future clinical use.

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