Intratumoral Neoadjuvant Immunotherapy Using IL-12 and Dendritic Cells Is an Effective Strategy To Control Recurrence of Murine Hepatocellular Carcinoma in Immunosuppressed Mice

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Liver transplantation is accepted as an effective therapy for hepatocellular carcinoma (HCC). However, recurrence is one of the most fatal complications. The aim of this study is to evaluate the efficacy of intratumoral immunotherapy using IL-12 gene therapy and dendritic cell injection for the purpose of effective treatment for HCC under conditions of immunosuppression. We found that the combined immunotherapy significantly induced sustained and high amounts of intratumoral IL-12 and IFN-γ proteins and that it induced high HCC-specific CTL activity under immunosuppression as compared with each monotherapy or control. The combined immunotherapy also exerted effective antitumor effects on the immunosuppressed host, resulting in significant suppression of growth of the s.c. established tumor and complete suppression of lung and liver metastasis, without rejection of a fully allogeneic skin graft. These antitumor effects were dependent on both T cells and NK cells. Noteworthy, the combined intratumoral immunotherapy and tumor resection (that is, neoadjuvant immunotherapy) resulted in achievement of tumor-free and long-term survival of the some immunosuppressed mice, even when the mice were challenged with i.v. injection of HCC at the time of tumor resection. In contrast, all of the mice treated with neoadjuvant immunotherapy using monotherapy or control therapy suffered from lung and liver metastasis. These results suggest that intratumoral neoadjuvant immunotherapy using IL-12 gene therapy and dendritic cell therapy is a potent effective strategy to control recurrence of HCC in patients after liver transplantation for HCC and may be applicable to general cancer treatment. The Journal of Immunology, 2010, 185: 698–708.

Cancer immunotherapy is the fourth most promising strategy for standard cancer therapies after operations, chemotherapy, and radiotherapy. Cancer immunotherapy intrinsically has memory responses in the long-term period mediated by adaptive immune responses, which is in contrast to operations, radiation, or chemotherapy. Therefore, application of cancer immunotherapy is a most expectative for cure of patients with advanced cancer. To date, many protocols of immunotherapy have been developed, and a number of protocols have been applied to human trials and therapeutic strategy (1, 2). However, the effect of the therapy is extremely limited, even in cases in which tumor-specific CTL responses are detected when end-stage patients who are burdened with a well-established tumor are treated with cancer immunotherapies (2). The reason why large tumors are difficult to eradicate is that the established large tumor can cause various adverse microenvironment for immune responses (3), including downregulation of MHC in tumor and tumor-associated stromal cells (4), production of TGF-β (4) and apoptosis-inducing factors (4), inhibition of dendritic cell (DC) differentiation and function (5), increment of regulatory T cells (6), prevention of CD8+ T cell recognition by immature myeloid cells (7), and immature microvessel formation preventing effective T cell infiltration (8). Therefore, reduction of absolute tumor volume to the level of subclinical disease may be required to obtain a stable and effective therapeutic response. In such a sense, development of neoadjuvant immunotherapy into standard cancer therapy may be a promising treatment.

Hepatocellular carcinoma (HCC) is one of the most frequent and malignant diseases worldwide. With an estimated 550,000 newly diagnosed cases per year, it is the third most common cause of deaths from cancer (9). Liver transplantation (LT) is widely accepted as the treatment of choice for end-stage liver failure, including early-stage HCC meeting the Milan criteria (a single tumor ≤5 cm in diameter or with ≤3 tumors all ≤3 cm in diameter), which is currently widely accepted as an effective way of selecting patients with HCC (10, 11). It was found that a significant proportion of patients with HCC who had tumors that were ≥5 cm could achieve long-term survival after LT in the context of multimodal adjuvant therapy (12). This previous finding led to the expansion of the indication of LT for HCC, including HCC exceeding the Milan criteria. However, it has been reported that cumulative HCC recurrence in patients meeting or exceeding the Milan criteria was 2.5 or 19.6% at 1 y and 2.5 or 36.6% at 3 y, respectively (13). When LT is performed as a therapy for HCC,
recurrence is one of the most fatal complications. The survival of recurrent cases after LT is significantly poor despite systemic chemotherapy (12). One of the reasons could be because of the decrease in cancer immunosurveillance by immunosuppressants (14, 15), Therefore, to prevent recurrence under conditions of immunosuppression after LT for HCC, we need to develop a new active strategy, which prevents distant micrometastases without affecting graft function.

IL-12 exerts a variety of immunomodulatory antitumor effects, including induction of IFN-γ secretion from T cells and NK cells (16), promotion of maturation of CTLs (16), and induction of angiogenic effects (17). A number of experimental studies have demonstrated that local or systemic treatment with rIL-12 protein mediates profound antitumor effects in vivo, causing suppression of growth of established tumors and their distant metastasis (16). However, human clinical trials with IL-12 monotherapy have shown limited efficacy in most instances (18), suggesting that additional modification in IL-12-based immunotherapy should be developed to enhance the clinical response. We have also previously reported that electroporation-mediated IL-12 gene therapy is effective not only in inhibiting the growth of a well-established s.c. HCC, but also in suppressing lung metastasis by NK and T cells, even in sufficiently immunosuppressed mice (19). However, this antitumor effect was mainly dependent on NK cells, and T cell contribution was relatively weak, which could be important for inducing long-lived memory T cell responses to tumors for efficient antitumor effects in cancer immunotherapy (20). In contrast, we and others have reported that intratumoral administration of DCs, which are the most potent and professional APCs (20) that can prime and control Ag-specific T cell responses (21), induces a significant T cell-dependent antitumor effect concomitant with a strong T cell response to tumor Ags (22, 23). The mechanism of the antitumor effect by intratumoral DC injection is believed to be as follows: injected DCs capture tumor Ags in the tumor site and migrate into lymph nodes to prime tumor-Ag-specific T cells (22). Therefore, this DC-based immunotherapy does not require that tumor Ags including peptide or tumor lysate be obtained (23). Considering the following features: 1) intratumoral administration of DC directly compensates for DC-defects in the tumor microenvironment (5); 2) the antitumor effect of intratumoral administration of DC is enhanced by an increment of local apoptotic tumor cells (22); 3) intratumoral IL-12 gene therapy exerts an antiangiogenic effect, which is mainly dependent on NK cells and by which tumor tissue undergoes necrosis (19); 4) IL-12 directly enhances T cell proliferation and function (24); and 5) IL-12 exerts various immunological adjuvant effects (25), intratumoral combined immunotherapy with IL-12 gene therapy and injection of DC warrant investigation.

In the current study, we investigated the efficacy of intratumoral combined immunotherapy with IL-12 gene therapy and intratumoral DC injection for local antitumor effects and antimetastatic effects against well-established HCCs in mice under conditions of immunosuppression by FK506, which is a calcineurin inhibitor (26). We also assessed the efficacy of intratumoral neoadjuvant immunotherapy using the combined immunotherapy involved with tumor resection for prevention of HCC recurrence.

Materials and Methods

Cell lines

MH134 (H-2b), a murine HCC cell line induced using carbon tetrachloride in C57Bl/6 mice, was obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer (Tohoku University, Miyagi, Japan), X5563 (H-2b), a plasmacytoma cell line derived from the C3H strain, was obtained from the Division of Molecular and Cellular Immunology, Medical Institute of Bioregulation (Kyushu University, Fukuoka, Japan).

Animals

Eight-week-old female C3H/HeN mice (H-2h) and BALB/c mice (H-2a) were purchased from Charles River Laboratories (Tokyo, Japan) and maintained under specific pathogen-free conditions in the animal facility at the Kyushu University Medical School. These experiments were approved by the Kyushu University Institutional Animal Care and Use Committee and conformed to the guidelines outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy for Sciences and published by the National Institutes of Health.

Plasmid DNA

IL-12 plasmid vector, designated pCAGGS-IL-12 for IL-12 gene therapy, and an empty plasmid vector, designated pCAGGS for controls, were used in the study. Details including the construction of the vectors are as previously described (27, 28).

Generation of bone marrow-derived DCs

Bone marrow-derived DCs (BM-DCs) were generated as we previously reported (23). On day 6, BM-DCs were collected and incubated with 0.5 μg/ml LPS (Sigma-Aldrich, St. Louis, MO) for 8 h. BM-DCs were then collected and treated with 50 μg/ml polymyxin B (Sigma-Aldrich) to neutralize the free LPS and carefully washed by PBS (Invitrogen, Paisley, Scotland) two times preinjection. The maturation status was confirmed by assessment of expression of stimulatory-related molecules (CD80, CD86, and ICAM-1) and I-Aβ using flow cytometry (data not shown) (23).

Murine tumor model

After MH134 cells were s.c. injected in the right flank of the C3H/HeN mice, 100 μg DNA solution at 1.0 μg/μl (pCAGGS-IL-12 or pCAGGS for controls) was injected into the established MH134 tumor, and electroporation was performed at the time when the tumor volume reached ~0.5 cm3 (this time point is defined as day 0) (19, 27, 28). The electroporation was performed as follows: a pair of electrode needles was inserted into the tumor to a depth of 5 mm to encompass the DNA injection sites, and electric pulses were delivered using an electric pulse generator (CUY-21; BEX, Tokyo, Japan) at the optimal condition: 150 V, 50 ms in duration, and 10 shocks (27). We confirmed that ~45% of the tumor cells in the in situ MH134 tumors were able to express enhanced GFP using pCAGGS-EGFP as a measure of the transfection efficiency (data not shown). On day 2 after the DNA injection, 1.0 × 106 BM-DC, which was suspended in 100 μl PBS or 100 μl PBS only for controls, was injected into the established s.c. MH134 tumor. From day 8 after the DNA injection, the mice were i.p. treated with 3 mg/kg FK506 (Astellas Pharma, Tokyo, Japan) every day. This dose was sufficient to prevent rejection of fully allogeneic skin grafts (19). On day 9 after the DNA injection, skin grafting was performed on the left flank of all mice using the allogeneic skin of BALB/c mice to assess the state of immunosuppression as previously described (19) (Fig. 1A). For a period of 28 d after the DNA injection, the tumor sizes were assessed using microcalipers, and the tumor volumes were calculated (29). For intratumoral neoadjuvant immunotherapy, we added two procedures to the protocol as described above: on day 9 after the DNA injection (on day 7 after DC injection), the primary established s.c. MH134 tumor was surgically resected, and then the mice were challenged to i.v. injection with 5 × 105 MH134 cells (Fig. 6A).

Histopathological analysis of metastatic lesions

The resected lungs and livers were fixed in 10% buffered formalin and embedded in paraffin. The paraffin-embedded sections were stained with H&E for histopathological analysis. The degree of metastasis was evaluated by the largest diameter (in micrometers) and the number of metastatic lesions at the maximum areas, which included a sagittal section through the left lobe of the lung and a horizontal section through the middle lobe of the liver.

ELISA for IL-12 and IFN-γ

Frozen specimens of the resected established s.c. HCCs were homogenized in an extraction buffer on ice and assayed for the heterodimeric IL-12 p70 (Pierce, Rockford, IL) and IFN-γ (BioSource International, Camarillo, CA) using an ELISA kit. Each cytokine concentration was divided by the total protein concentration as previously described (19).
Assay system for the secondary CTL response

The assay for the secondary CTL response was performed as previously described (19, 23).

Ab-mediated depletion of immune cell subsets in vivo

Anti-CD4 or anti-CD8 mAb, which was derived from GK1.5 or 53-6.72 hybridoma cells, respectively, was given i.p. (250 μg/dose) for CD4+ T cells or CD8+ T cell depletion. The CD4+ or CD8-depleting Ab eliminated the CD4+CD11c+ DC (30) or CD8a+CD11c+ DC (data not shown), respectively. Although the CD8a+CD4+ CD11c+ is exclusively crucial for crosspriming of CD8 T cells, other DCs can prime CD4 T cells. Therefore, we believe that there are no technical concerns for these Ab-mediated depletion studies in which CD4 T cell or CD8 T cell response is required to be eliminated. Antiasialo GM1 antiserum (Wako Bioproducts, Tokyo, Japan) was given i.p. (50 μg/dose) for NK cell depletion. Flow cytometry confirmed a >98% depletion of the target cells. Elimination of CD4+ T cells, CD8+ T cells, or NK cells in mice with an established MH134 was performed by i.p. injection on days −2, −1, and 0 and once every other day thereafter for an additional 26 d (16 times in total).

Skin grafting

Skin grafting was performed as previously described (19, 31, 32). Briefly, square full-thickness skin grafts (1 cm²) were prepared from the trunk skin of BALB/c mice. Graft beds (1 cm²) were prepared on the left flank of the C3H mice. The donor grafts were fixed to the graft bed with eight interrupted sutures of 6-0 Prolene thread (Johnson & Johnson, North Ryde, Australia) and covered with protective tape. The first inspection was conducted on day 7 after skin grafting followed by daily inspection. Grafts were considered as rejected at the time of complete sloughing or shrinking or when they formed a dry scar. The C3H-derived skin grafts were accepted in the syngeneic C3H mice without FK506 administration, whereas the BALB/c-derived allogeneic skin grafts showed massive chronic inflammatory infiltrates and were rejected on approximately day 7 when FK506 was not administered (Table I, Experiment 1). However, the allogeneic skin grafts could be successfully maintained during administration of FK506 for at least 40 d (Fig. 7, Table I, Experiments 2 and 3, and data not shown). The skin grafts eventually showed a reddish appearance and exhibited ulceration owing to massive chronic inflammatory cell infiltration when FK506 was abruptly withdrawn postadministration for 1 mo (33–35) (data not shown). Calcineurin inhibitors not only inhibit the proliferation of alloreactive naïve T cells but also inhibit the apoptosis of alloreactive activated T cells (36). Therefore, the alloreactive T cells may proliferate and infiltrate into the grafted allogeneic donor skin and cover the protective tape. The first inspection was conducted on day 7 after skin grafting followed by daily inspection. Grafts were considered as rejected at the time of complete sloughing or shrinking or when they formed a dry scar. The C3H-derived skin grafts were accepted in the syngeneic C3H mice without FK506 administration, whereas the BALB/c-derived allogeneic skin grafts showed massive chronic inflammatory infiltrates and were rejected on approximately day 7 when FK506 was not administered (Table I, Experiment 1). However, the allogeneic skin grafts could be successfully maintained during administration of FK506 for at least 40 d (Fig. 7, Table I, Experiments 2 and 3, and data not shown). The skin grafts eventually showed a reddish appearance and exhibited ulceration owing to massive chronic inflammatory cell infiltration when FK506 was abruptly withdrawn postadministration for 1 mo (33–35) (data not shown). Calcineurin inhibitors not only inhibit the proliferation of alloreactive naïve T cells but also inhibit the apoptosis of alloreactive activated T cells (36). Therefore, the alloreactive T cells may proliferate and infiltrate into the grafted allogeneic donor skin postwithdrawal of FK506, resulting in rejection (data not shown). These observations suggest that long-term administration of FK506 does not result in tolerance induction and that the grafted skin can act as a biomarker for sufficient immunosuppression (34).

Statistical analysis

All data are expressed as means ± SEM. Statistical evaluations of numerical variables between different groups were mainly performed using the Mann-Whitney U test. Differences in tumor growth were statistically analyzed using a one-way ANOVA. Significant differences were determined by a post hoc test, Fisher’s exact probability test. The survival data were statistically analyzed by log-rank test. Significance was defined as p < 0.05. The analyses were performed with the use of Stat View software (version 5.0, Abacus Concepts, Berkeley, CA) or the use of GraphPad Prism 4 software (version 4, GraphPad, San Diego, CA).

Results

Combined immunotherapy using intratumoral IL-12 gene therapy and intratumoral DC injection induces an enhanced antitumor effect on primary established HCC under immunosuppression by FK506

We previously found that electroporation-mediated IL-12 gene therapy is effective not only in treating local tumors but also in suppressing lung metastasis, and this antitumor effect was mainly dependent on NK cells (19). To enhance the T cell response to HCC, we combined IL-12 gene therapy with intratumoral administration of DC. We first examined the antitumor effect of the combined immunotherapy using intratumoral IL-12 gene therapy with the murine IL-12 plasmid vector (designated as pCAGGS-IL-12) and intratumoral injection of murine BM-DCs (combined therapy group) and assessed the primary established MH134 tumor under immunosuppression by FK506 (Fig. 1A). This was compared with therapies using pCAGGS-IL-12 and PBS (IL-12 monotherapy group), control empty plasmid vector (designated as pCAGGS) and BM-DCs (DC monotherapy group), and pCAGGS and PBS (control group). The growth of MH134 tumor in the combined therapy group was significantly inhibited, even if the mice were under immunosuppression by FK506, as compared with the IL-12 monotherapy group (p < 0.05), DC monotherapy group (p < 0.01), and control group (p < 0.01; Fig. 1). All skin grafts were maintained without any signs of rejection, suggesting that the dose of FK506 was sufficient to prevent rejection of a fully allogeneic graft (Table I, Experiments 1 and 2), and combined cancer immunotherapy did not disturb the control of allograft rejection by FK506.

Intratumoral administration of DC in addition to IL-12 gene therapy maintains the expression of biologically active IL-12 and IFN-γ proteins in tumors in immunosuppressed mice

We previously reported that intratumoral IL-12 gene therapy (IL-12 monotherapy) resulted in suppression of growth of primary established s.c. HCC, even under immunosuppression through the...
cooperation of three mechanisms: the suppression of neovascularization of HCC, generation of tumor-specific CTLs, and enhancement of infiltration of various lymphocytes by local elevation of bioactive IL-12 (19) and IFN-γ, which is considered to be the most important factor in IL-12 therapy (37). Therefore, we investigated the amount of intratumoral IL-12 and IFN-γ proteins in this experimental setting. On day 7, the expression of intratumoral IL-12 protein in the combined therapy group was significantly higher compared with the DC monotherapy group (p < 0.05) and control group (p < 0.05), but there was no significant difference between the combined therapy group and IL-12 monotherapy group (Fig. 2A). However, on day 14, the expression of intratumoral IL-12 protein in the combined therapy group was significantly higher compared with the IL-12 monotherapy group (p < 0.05), the DC monotherapy group (p < 0.05), and the control group (p < 0.05) (Fig. 2A). Furthermore, we examined upregulation of intratumoral IFN-γ protein as an indication of biological activity of IL-12 protein. The degree of expression of intratumoral IFN-γ protein in each group was correlated with that of IL-12 protein (Fig. 2B). Therefore, biologically active IL-12 and IFN-γ proteins could be maintained at significantly higher amounts in the primary established MH134 tumors in the combined therapy group than those in the other groups.

**Combined immunotherapy can effectively control spontaneous lung and liver metastasis in tumor-burdened mice under immunosuppression by FK506**

In cancer immunotherapy, it is important to control metastasis for obtaining a curative effect or prolonging survival. Therefore, we examined the antimitastatic effects of combined immunotherapy for spontaneous lung and liver metastasis using the model as described in Fig. 1. The mice were sacrificed on day 28, and the degree of metastasis in lungs and livers was evaluated. Spontaneous lung metastasis was observed in five out of eight mice (63%) in the IL-12

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**FIGURE 2.** Maintained expression of IL-12 and IFN-γ proteins in tumors in immunosuppressed mice that had combined immunotherapy using IL-12 and DCs. Mice with an established s.c. MH134 tumor in each group (combined therapy group: closed bars, n = 3; IL-12 monotherapy group: hatched bars, n = 3; DC monotherapy group: gray bars, n = 3; control group: open bars, n = 3) were sacrificed on day 7 (left panels) or on day 14 (right panels), and the s.c. tumors were resected. The protein levels of intratumoral IL-12 (A) and IFN-γ (B) were estimated by ELISA. The data are indicated as mean protein levels ± SEM and are representative of three independent experiments. Statistical significance by the Mann-Whitney U test (p < 0.05) is indicated in the bar graphs.

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**Table I. Survivals of skin grafts**

<table>
<thead>
<tr>
<th>Intratumoral Treatment</th>
<th>Skin Graft</th>
<th>FK506</th>
<th>Survival Time of Skin Graft (d)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>n Value</td>
<td>IL-12</td>
<td>DC</td>
<td>Donor</td>
</tr>
<tr>
<td>Experiment 1</td>
<td>3</td>
<td>C3H</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>BALB/c</td>
<td>–</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>8</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>BALB/c</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>BALB/c</td>
<td>+</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>9</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>BALB/c</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>+</td>
<td>+</td>
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</table>

*Number of days from skin grafting to when the recipients were sacrificed or died after skin grafting.

+ Experiment 1 was obtained using IL-12 protein in each group; Experiment 2 was obtained using IL-12 protein in each group; Experiment 3 was obtained using IL-12 protein in each group. The BALB/c trunk skins were grafted on C3H-recipient mice, which were indicated in Figs. 6 and 7.

**The BALB/c trunk skins were grafted on untreated C3H-recipient mice.**
monotherapy group, eight out of eight mice (100%) in the DC monotherapy group, and eight out of eight mice (100%) in the control group (Fig. 3A–C). Spontaneous liver metastasis was observed in six out of eight mice (75%) in the IL-12 monotherapy group, eight out of eight mice (100%) in DC monotherapy group, and eight out of eight mice (100%) in the control group (Fig. 3D–F). Interestingly, no spontaneous lung and liver metastases could be detected in the combined therapy group, even if the mice were under immunosuppression by FK506. These findings suggest that the combination of intratumoral IL-12 gene therapy and DC injection could additively or synergistically enhance the antimetastatic effect for spontaneous lung and liver metastasis, even if the mice were under immunosuppression by FK506.

**Combined immunotherapy using intratumoral DC injection and IL-12 gene therapy enhances and maintains systemic MH134-specific CTL responses in mice under immunosuppression by FK506**

CTLs play an important role in suppressing tumor progression and metastasis (38, 39), and a maintained tumor Ag-specific CTL response is important for an enhanced antitumor effect (40, 41). To assess the mechanism by which combined immunotherapy induces enhanced antitumor and antimetastatic effects, we investigated systemic MH134-specific CTL activities. As expected, the CTL response from mice in the combined therapy group was higher than that in the IL-12 monotherapy group, the DC monotherapy group, and the control group on day 7 after the DNA injection (Fig. 4A). Furthermore, on day 14 after the DNA injection, the CTL response in the combined therapy group was maintained and significantly higher than that in the IL-12 monotherapy group, the DC monotherapy group, and the control group (Fig. 4B). In additional experiments, it was confirmed that such an augmented CTL response was MH134 specific, because the generated CTLs had no killing toward the third-party plasmacytoma X5563, derived from the C3H strain (Table II). Therefore, a systemic MH134-specific CTL response could be maintained at a significantly higher level in the combined therapy group than that in the IL-12 or DC monotherapy group, even if the mice were under immunosuppression by FK506.

**T cells and NK cells are indispensable for inhibition of primary tumor growth and the antimetastatic effect of combined immunotherapy in an immunosuppressed host**

To determine which subsets of cells are essential for control of local tumor growth and antimetastatic effects in combined immunotherapy using IL-12 and DCs under immunosuppression by FK506, we conducted depletion studies of immune cell subsets by administration of depleting Abs against CD8+ T cells, CD4+ T cells, and NK cells in mice with an established MH134 tumor treated by combined immunotherapy. Depletion of CD8+ T cells, CD4+ T cells, or NK cells abrogated the inhibition of growth of primary s.c. MH134 tumors in combined immunotherapy (Fig. 5A). Furthermore, depletion of CD8+ T cells, CD4+ T cells, or NK cells cancelled the antimetastatic effects of the immunotherapy for spontaneous lung and liver metastasis under immunosuppression by FK506. Both spontaneous lung and liver metastasis were

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**FIGURE 3.** Effective control of spontaneous lung and liver metastasis in combined immunotherapy under conditions of immunosuppression. Mice with an established s.c. MH134 tumor in each indicated group (as described in Fig. 1) were sacrificed on day 28, and metastasis of MH134 in the lungs (A–C) and livers (D–F) was microscopically evaluated with H&E staining. The bar graphs (combined therapy group: closed bars, $n = 8$; IL-12 monotherapy group: hatched bars, $n = 8$; DC monotherapy group: gray bars, $n = 8$; control group: open bars, $n = 8$) show the largest diameter (in micrometers) of metastatic foci (A, D) and the number of metastatic lesions (B, E). The data shown are mean value ± SEM and representative of three independent experiments. Statistical significance by the Mann-Whitney $U$ test ($p < 0.05$; $p < 0.01$) is indicated in the bar graphs. The frequency of metastasis (number of mice with metastasis/total number of experimental animals) is indicated above the bars in each group (B, E). The photographs show representative microscopic findings of the resected lungs (C, original magnification $\times 200$) and livers (F, main photographs, original magnification $\times 200$; insets, $\times 400$) in each group. Arrows indicate the typical findings of microscopically metastatic foci.
observed in three out of three mice (100%) in groups with CD8+ T cell, CD4+ T cell, and NK cell depletion (Fig. 5B–E). There were no significant differences in the mean largest diameter and number of spontaneous lung and liver metastases among each group with CD8+ T cell, CD4+ T cell, and NK cell depletion (Fig. 5B–E). Data from the CTL response and these findings suggested that intratumoral DC therapy in addition to IL-12 gene therapy could augment the T cell response and both local tumor growth suppression and antimetastatic effects of the combined immunotherapy were dependent on coordination of the T cell and NK cell response.

Intratumoral neoadjuvant immunotherapy using injection of DC and IL-12 gene therapy controls HCC recurrence and improves the survival of immunosuppressed mice

The purpose of this study was to develop a new effective therapy to prevent recurrence of HCC under conditions of immunosuppression after LT for HCC, resulting in a bona fide curative operation. We designed a neoadjuvant immunotherapy model using intratumoral DC injection and intratumoral IL-12 gene therapy. We added two procedures to the protocol as described above (Fig. 1A): on day 9 after the DNA injection, when there were no differences among the volumes of the primary established s.c. HCC in each group, the primary established s.c. HCC was surgically resected, and then the mice were challenged to i.v. injection with MH134 cells (Fig. 6). The i.v. injection of MH134 cells was performed for the purpose of confirming the antimetastatic effect by overloading with circulating HCC and for mimicking the clinical situation in which dissemination to circulation by manipulation during operation of LT for HCC. Lung and liver metastases were evaluated on day 28. Lung metastasis was observed in three out of three mice (100%) in the IL-12 monotherapy group, two out of three mice (67%) in the DC monotherapy group, and three out of three mice (100%) in the control group. Liver metastasis was observed in three out of three mice (100%) in the IL-12 monotherapy group, three out of three mice (100%) in the DC monotherapy group, and three out of three mice (100%) in the control group (Fig. 6). No lung or liver metastasis could be detected in the combined therapy group, even if the mice were under immunosuppression by FK506. Furthermore, we investigated the survival of the mice in each experimental group. All of the mice in the control group, the IL-12 monotherapy group, and the DC monotherapy group died within 60 d, whereas six out of nine mice in the combined immunotherapy group were alive on day 70 (Fig. 7A). We confirmed the cause of death was tumor metastasis involving lung, liver, and lymph node (Fig. 7B and data not shown). In addition, we did not detect tumor metastasis involving lung, liver, and lymph node in the long-survived mice in the combined therapy group with histological examination at the end of the experiment (data not shown). None of the skin grafts were rejected, suggesting that the immunosuppression was sufficient to control rejection and was not hampered by the immunotherapy (Fig. 7B, Table I, Experiment 3). These findings suggest that both intratumoral IL-12 gene therapy and intratumoral injection of DC are required to obtain antimetastatic effects for HCC under immunosuppression by FK506 in neoadjuvant immunotherapy.

Discussion

In this study, we demonstrated that combined immunotherapy using intratumoral IL-12 gene therapy and intratumoral injection of DCs exerted a strong antitumor effect, including suppression of local tumor growth and inhibition of lung and liver metastases in sufficiently immunosuppressed mice by FK506. In addition, intratumoral neoadjuvant immunotherapy using the combined immunotherapy controlled HCC recurrence of immunosuppressed mice without rejection of allogeneic skin grafts. To our knowledge, this is the first report of intratumoral neoadjuvant immunotherapy using a combination of electroporation-mediated intratumoral IL-12 gene therapy and DC-based immunotherapy under conditions of immunosuppression.

In our institution, LT was carried out for 90 patients with HCC, and 53 (59%) patients preoperatively exceeded the Milan criteria. HCC recurrence was recognized in 14 (16%) out of 90 patients, and 13 (93%) out of 14 patients with recurrence exceeded the Milan criteria. The overall survival rates in patients meeting or exceeding the Milan criteria was 100 or 85.5% at 1 y, 100 or 68.2% at 3 y, and 100 or 68.2% at 5 y, respectively (data not shown) (42). Therefore, we believe that intratumoral neoadjuvant immunotherapy using combined immunotherapy is a promising strategy for increasing the rate of curative operations in LT for HCC and for improving the prognosis of patients with HCC exceeding the Milan criteria.

### Table II. Specificity of secondary CTL response

<table>
<thead>
<tr>
<th>Target Cell</th>
<th>Percent Specific Lysis at E:T Ratio*</th>
<th>Combined Therapy</th>
<th>IL-12 Monotherapy</th>
<th>DC Monotherapy</th>
<th>Control</th>
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<tr>
<td>Day 7†</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>MH134</td>
<td>14.2</td>
<td>11.2</td>
<td>4.5</td>
<td>0.0</td>
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<td>X5563</td>
<td>1.3</td>
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<td>0.6</td>
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<td>Day 14‡</td>
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<tr>
<td>MH134</td>
<td>22.8</td>
<td>11.0</td>
<td>4.9</td>
<td>0.0</td>
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<tr>
<td>X5563</td>
<td>1.9</td>
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*Data indicate mean percent specific lysis of triplicate wells at an E:T ratio of 80:1.

†On day 7 and day 14, splenocytes were harvested from mice in the indicated group as described in Fig. 1. Effector cells were generated as described in Fig. 4, and a [51Cr] release assay for MH134 or X5563, a third-party control, was also performed.
Although early clinical trials have shown promising results (43, 44), during the last decade, clinical trials of DC-based immunotherapy have shown a relatively limited clinical outcome against intractable malignancies (2, 45). Human clinical trials with IL-12 monotherapy and other immunotherapies have also shown limited efficacy in most instances (18, 46). In these clinical trials, immunotherapy has been used to treat patients in advanced stages of cancer. Because well-established cancers display multiple immunosuppressive mechanisms to evade an immune response, it may be difficult to eradicate a solid cancer completely (5, 8, 47, 48). These features are also true of experimental rodent models; for example, intratumoral DC treatment can frequently cure mice with established murine B16-F1 melanoma, of which the largest diameter is <0.7 cm, but it is difficult to cure mice with B16-F1, of which the diameter is >0.7 cm (23)(S. Okano, unpublished observation). Encouraging experimental evidence suggests that the systemic highly immunosuppressive status can be normalized by resection of the established tumor (49). In fact, intratumoral combined immunotherapy could not eradicate large established HCC even with a lasting HCC-specific CTL response and high IL-12-bioactivity, but, just in a neoadjuvant setting, the therapy could achieve tumor-free and long-term survival of the mice.

Our combined immunotherapy targeted the primary tumor site for the following reasons: 1) it is difficult to obtain a sufficient Ag or autologous tumor lysate for DC immunotherapy in the preoperative period when performing neoadjuvant immunotherapy and also to take measures against Ag loss variants; 2) intratumoral IL-12 gene therapy-mediated tumor necrosis (19) can be an abundant source of tumor Ag for DCs (22); 3) intratumoral IL-12 gene therapy can avoid adverse effects caused by systemic IL-12 treatment; and 4) local IL-12 effectively recruits intratumoral NK cells (19) that mutually interact with DCs, and this interaction enhances the function of both NK cells and DCs, resulting in augmentation of the T cell response (50). We demonstrated that the intratumoral combined immunotherapy exerted an enhanced and lasting HCC-specific CTL response associated with enhanced and sustained IL-12–IFN-γ-production in HCC. The enhanced CTL response may be an important factor for eliciting an enhanced antimetastatic effect as compared with monotherapy. Tatsumi et al. (51) reported that s.c. vaccination of tumor lysate-pulsed DCs at a 7-d period over a 3-wk period and systemic IL-12 administration for 5 consecutive days after each immunization suppresses local s.c. HCC, and IL-12 enhances CTL responses mediated by DCs, although they did not evaluate the antimetastatic effect. We have also found the following: 1) intratumoral IL-12 gene therapy elicited an HCC-specific CTL response probably through host-derived DCs (19); 2) intratumoral DC monotherapy could eradicate not only treated established B16-F1 but also distant B16-F1 melanoma; however, this
was not the case for vaccination of tumor lysate-pulsed DCs in a distant site (23) (S. Okano, unpublished observation); 3) intratumoral DC monotherapy could eradicate B16-F1 in IL-12Rβ2 (high-affinity IL-12R)-deficient mice at the same rate as in wild-type mice (S. Okano, unpublished observation), suggesting that intratumoral DC therapy does not absolutely require IL-12 and that concomitant intratumoral IL-12 gene therapy can synergistically augment the antitumor effect of DC therapy. It is likely that targeting tumor sites can also have a beneficial influence on the enhanced antitumor effects in our protocol. In fact, at least with regard to the response of NK cells, contribution of NK cells, from which an early source of IFN-γ is strictly required for Th1 response (50, 52), was essential in our intratumoral combination therapy (Fig. 5). In contrast, the contribution of NK cells was small in Tatsumi’s et al. protocol (51).

CD4+ T cells, CD8+ T cells, or NK cells can independently exert antitumor effects with different mechanisms (50, 53). If each of the cell types independently exerts an antitumor immune response in our combined immunotherapy, a depletion study should show a partial decrease in the antitumor effect. However, depletion of a single subset among CD4+ T cells, CD8+ T cells, and NK cells abrogated the antitumor effect, including inhibition of local primary tumor growth and suppression of distant metastasis in combined immunotherapy using IL-12 and DCs (Fig. 5). These findings suggest that mutual interactions among all of the subsets are required for inducing sufficient antitumor effects. Because the depletion was performed preimmunotherapy in our protocol, these immune subsets may have an essential role in the T cell-priming phase. We previously reported that in IL-12 gene therapy alone under immunosuppression by FK506, NK cells played a critical role, and contribution of T cells is partial for the antitumor effect, even in the successful priming of the HCC-specific CD8+ T cell response (19). Therefore, we believe that it is important that intratumoral DC therapy in addition to IL-12 gene therapy augment the T cell response through cross-talk of DC-NKT cells in the T cell-priming phase, resulting in sufficient antimetastatic effects.

FK506 forms a complex with FK506-binding protein. This complex can bind to calcineurin and block its ability to activate NFAT1. As a result, the inductions of several lymphokines, including IL-2, are inhibited (26). Regarding the priming of naive T cells, FK506 is able to block the priming (proliferation) of naive T cells via inhibition of IL-2 production. Gardner et al. (35) previously suggested that calcineurin inhibitors may arrest the immune response at an early stage and maintain this situation while they are still being administered. When the drug treatment is stopped and the drug effects have worn off, the immune response begins (reversible effect). Regarding activated effector or memory T cells, FK506 prevents apoptosis of activated T cells and is unable to block the proliferation of activated T cells (54). Therefore, it is possible that once T cells are primed to resist apoptosis and suppress their effector function under FK506 administration, this leads to the accumulation of tumor-specific T cells in FK506-injected mice for long periods. In line with this hypothesis, we

**FIGURE 6.** Inhibition of recurrence of HCC with intratumoral neoadjuvant immunotherapy using injection of DC and IL-12 gene therapy in immunosuppressed mice. The mice with an established s.c. MH134 tumor were treated with pCAGGS-IL-12 and BM-DCs (combined therapy group; closed bars; n = 3), pCAGGS-IL-12 and PBS (IL-12 monotherapy group; hatched bars; n = 3), pCAGGS and BM-DCs (DC monotherapy group; gray bars; n = 3), and pCAGGS and PBS (control group; open bars; n = 3). From day 8, the mice were treated with FK506 every day. On day 9, the primary established MH134 tumor was surgically resected, skin grafting from BALB/c mice was performed, and then the mice were challenged with an i.v. injection with MH134 cells. A, The overall experimental design in the combined therapy group is illustrated. The mean largest diameter (left panels) and number (right panels) of lung (B, C) and liver (D, E) metastatic lesions were evaluated. The data shown are mean ± SEM. Statistical significance by the Mann-Whitney U test (p < 0.05) is indicated in the bar graphs. The frequency of metastasis (number of mice with metastasis/total number of experimental animals) is indicated above the bars in each group (C, E). The data are representative of two independent experiments.
previously observed enhanced MH134-specific CTL activity in mice under FK506 administration compared with immunocompetent mice during intratumoral IL-12 gene therapy (19). In the present experimental setting, administration of FK506 was started 8 d after the combined immunotherapy. In the general immune response at that time, the number of activated T cells is at its peak, and the activated T cells undergo apoptosis thereafter (contraction phase). In contrast, the alloresponse to the donor graft is only in the priming phase. Considering the reciprocal effects of FK506 on the priming of naive T cells and apoptosis of activated T cells, the protocol in the current study may be suitable for maximal exertion of the tumor-specific immune response for the long term while preventing the rejection of donor grafts. This hypothesis is supported by our observation that enhanced MH134-specific CTL activity was detected on day 14 compared with day 7 (Fig. 4, Table II), resulting in complete and long-lasting antimetastatic effects (Figs. 6, 7) while maintaining the skin graft (Table I, Experiment 3).

The preoperative period is limited for performing immunotherapy. Therefore, it is noteworthy that it takes only 1 wk to perform intratumoral neoadjuvant immunotherapy with our combination therapy for a sufficient antimetastatic effect, even under immunosuppression. In addition, with LT for HCC, initial metastases are found in extrahepatic sites rather than in the liver, which is the common initial site of recurrence posthepatectomy for HCC. Therefore, it is an important finding that lung metastasis was suppressed in combined immunotherapy. However, adverse effects related to intratumoral injection should be considered in this protocol. In clinical settings, we have already used technical equipment for direct approaches to tumor sites, such as radiofrequency ablation therapy. Recently, a new device, the bipolar radiofrequency ablation device, has been developed (55). This new device has two electrodes and is able to not only turn on the power for liver nodules, such that larger tumor nodules can be treated, but also turn on the power for electroporation. In this study, we separately performed electroporation-mediated intratumoral IL-12 gene therapy and DC injection via the intratumoral route. For intratumoral IL-12 gene therapy, administration of an adenoviral vector encoding the IL-12 gene may be a safe and alternative tool (56, 57), whereby an additional device or procedure is not needed, and the administration has already been assessed for safety (57). We may be able to use injection of plasmid DNA encoding IL-12 alone because of the long-lasting IL-12 production achieved by such injections, which is caused by autocrine or paracrine positive feedback mechanisms involving several cell populations, including NK cells (58). Alternatively, we may be able to use DCs engineered to produce IL-12 (59–68) because previous studies have shown that the transduced IL-12 can augment the DC function to activate T cells and NK cells (65, 68, 69), IL-12 gene-transduced DC injection via an intratumoral route exerts superior antitumor effects in preclinical settings (61, 62), and phase 1 clinical trials of various advanced digestive tumors including HCC, in which the treatment was feasible and well tolerated, achieved one partial response and two stable disease among 17 patients (70, 71).

In our previous report (19), we also focused on the treatment target of recurrent HCC, and we are also interested in the effect of this combined immunotherapy on the donor graft, which is maintained by FK506. We examined this point using our skin graft model and found the combined immunotherapy did not hamper the suppression of graft rejection by FK506, even when the targeting tumor had existed just beneath the skin graft (Supplemental Fig. 1). Therefore, the combined immunotherapy may also have potential for treatment of recurrent HCCs in the donor liver graft in clinical settings. Further extensive preclinical studies should be performed before clinical application.

In conclusion, intratumoral neoadjuvant immunotherapy has the potential to become a new effective therapeutic strategy to contribute to the improvement of long-term survival after LT for HCC. In addition, based on careful medical ethics, preoperative active immunotherapy and operations targeting the primary cancer as a total cancer therapy should be applied to the clinical setting in the same manner as neoadjuvant chemoradiotherapies.

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Disclosures
The authors have no financial conflicts of interest.

References


