Administration of Interleukin-12 Enhances the Therapeutic Efficacy of Dendritic Cell-based Tumor Vaccines in Mouse Hepatocellular Carcinoma

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ABSTRACT

Dendritic cells (DCs) are potent antigen-presenting cells that are capable of priming systemic antitumor immune response. Here, we evaluated the combined effectiveness of tumor lysate-pulsed DC immunization and interleukin (IL-12) administration on the induction of antitumor immunity in a mouse hepatocellular carcinoma (HCC) model. Mouse DCs were pulsed with lysate of BNL 1ME A.7R.1 (BNL), a BALB/c-derived HCC cell line, and then injected into syngeneic mice in combination with systemic administration of IL-12. Lymphocytes from mice treated with BNL lysate-pulsed DCs and IL-12 showed stronger cytolytic activity and produced higher amounts of IFN-γ than from those mice treated with BNL lysate-pulsed DCs alone. Although immunization with BNL lysate-pulsed DCs alone did not lead to complete regression of established tumors, it significantly inhibited tumor growth compared with vehicle injection. Importantly, the combined therapy of BNL lysate-pulsed DCs and IL-12 resulted in tumor rejection or significant inhibition of tumor growth compared with mice treated with BNL lysate-pulsed DCs alone. In vivo lymphocyte depletion experiments demonstrated that this combination was dependent on both CD8⁺ and CD4⁺ T cells, but not natural killer cells. These results demonstrated that IL-12 administration enhanced the therapeutic effect of immunization of tumor lysate-pulsed DCs against HCC in mice. This combination of IL-12 and DCs may be useful for suppressing the growth of residual tumor after primary therapy of human HCC.

INTRODUCTION

HCC is one of the most common cancers in the world. Although HCC patients undergo medical and surgical treatment for primary tumors, intrahepatic recurrence and extrabiliary recurrence occur frequently, limiting patient survival (1–3). To improve prognosis, some way must be found to prevent the occurrence of second primary tumors. Because autologous HCC cells can be easily obtained at the time of initial therapy, a tumor-derived vaccine may be an attractive strategy to induce antitumor immunity against HCC.

DCs are potent antigen-presenting cells that can elicit primary immune response and boost secondary immune response to foreign antigens (4, 5). In a variety of settings, these specialized antigen-presenting cells can induce both the generation and proliferation of specific CTLs and Th cells via antigen presentation by MHC class I and class II molecules, respectively. Because of these properties, much attention has been directed toward the use of DCs in vaccine strategies for the treatment of cancer. In this regard, DCs pulsed with tumor-associated antigens in various forms, including whole cell lysate (6–9), peptides (10, 11), proteins (12), RNA (13), or DNA (14, 15), have been studied for antitumor effects in experimental tumor models. In these models, immunization with tumor antigens presented by DCs has shown much promise in effectively priming the cellular immune response as well as in eliciting tumor regression in vivo. Recently, two clinical applications using DC-based tumor vaccines have been reported (16, 17). One is for B-cell lymphoma patients, and the other is for melanoma patients. Although antitumor cellular immune responses could be induced by DC vaccination in all patients, clinical objective responses were limited in both tumor models. Thus, a new strategy for DC-based tumor vaccines is expected to improve the clinical effectiveness of this treatment.

IL-12 exhibits a number of important biological activities, including the ability to enhance NK and CTL activities (18–20) and to direct a cell-mediated immune response by generating a Th1-type immune response and inhibiting differentiation of Th2-type lymphocytes (21, 22). Recent reports demonstrated that IL-12 administration could enhance the stimulatory capacity of bone marrow-derived DCs in vitro (23) and that IL-12 in conjunction with DCs could enhance the antiviral CTL response in vitro (24). On the basis of these findings, we decided to evaluate the systemic administration of IL-12 for its capacity to enhance tumor lysate-pulsed DC activity in promoting therapeutic immunity in vivo in a mouse HCC tumor model.

In the present study, we have demonstrated that IL-12 can potentiate the antitumor effects of tumor lysate-pulsed DCs in vivo during treatment of mouse HCC tumors. This study suggests that immunization of tumor lysate-pulsed DCs in combination with administration of IL-12 may be useful for suppressing the growth of recurring tumors after primary therapy of human HCC.

MATERIALS AND METHODS

Mice. Female BALB/c mice were purchased from Shizuoka Experimental Animal Laboratory (Shizuoka, Japan) and used at 6–8 weeks of age. All animals were maintained in microisolation cages and handled under aseptic conditions. Procedures were performed according to approved protocols and in accordance with recommendations for the proper care and use of laboratory animals.

Cell Lines and Culture. BNL 1ME A.7R.1 (BNL), a mouse HCC cell line, was kindly provided by Dr. Takashi Tsuruo (Institute of Molecular and Cellular Bioscience, University of Tokyo, Tokyo, Japan) and maintained in RPMI 1640 (Nikken-Seibutsu) supplemented with 10% heat-inactivated FBS, antibiotics, and antimitotics (Life Technologies, Inc., Gaithersburg, MD) in a humidified atmosphere of 5% CO₂ at 37°C. Colon26, a mouse colon adenocarcinoma cell line, was kindly provided by Dr. Takashi Tsuruo (Institute of Molecular and Cellular Bioscience, University of Tokyo, Tokyo, Japan) and maintained in DMEM (Nikken-Seibutsu, Kyoto, Japan) supplemented with 10% heat-inactivated FBS, antibiotics, and antimitotics (Life Technologies, Inc., Gaithersburg, MD) in a humidified atmosphere of 5% CO₂ at 37°C. These tumor cell lines are BALB/c syngeneic.

Generation of DCs in Vitro from Bone Marrow. The procedure used in this study was described previously by Inaba et al. (25), with some minor modifications. Briefly, bone marrow was flushed from femurs and tibias and depleted of RBCs with ammonium chloride. Bone marrow cells were depleted from lymphocytes, granulocytes, and macrophages using a mixture of monoclonal antibody and rabbit complement. The monoclonal antibodies were 2.43 (anti-
RESULTS

Cytolytic Activity against Mouse HCC Cells and IFN-γ Production. To examine whether or not immunization of tumor lysate-pulsed DCs plus IL-12 induces systemic cytolytic activity of lymphocytes against parental BNL cells, mice were immunized with either BNL lysate-pulsed DCs plus IL-12, BNL lysate-pulsed DCs alone, IL-12 alone, or PBS. Spleen cells obtained from mice at 7 days after the final immunization were cocultured with MMC-treated BNL cells for 7 days. As shown in Fig. 1A, splenocytes from mice treated with BNL lysate-pulsed DCs showed cytolytic activity against BNL cells, whereas those from mice treated with IL-12 did not. Importantly, splenocytes from mice treated with both BNL lysate-pulsed DCs and IL-12 showed stronger cytolytic activity than those from mice treated with BNL lysate-pulsed DCs alone. This cytolytic activity was specific for BNL because splenocytes did not kill Colon26 colon cancer cells (Fig. 1B). Similar results were obtained in the three experiments. These results demonstrated that BNL lysate-pulsed DC immunization induced cytolytic activity of lymphocytes against BNL cells and that IL-12 enhanced the cytolytic activity induced by immunization with BNL lysate-pulsed DCs. Because IFN-γ is a Th1-associated cytokine critically involved in the development of cell-mediated immune response, we also analyzed the IFN-γ production released in an in vitro recall response to BNL tumor from splenocytes. Splenocytes obtained from mice treated with the combination therapy produced substantial amounts of IFN-γ (>100 units/ml), whereas those from mice treated with BNL lysate-pulsed DCs alone produced small amounts of IFN-γ (33 units/ml), and those from mice treated with IL-12 alone or PBS alone produced IFN-γ at levels ranging from 5 units/ml to undetectable (Fig. 1C). These results demonstrated that systemic administration of IL-12 enhanced IFN-γ production induced by DC immunization.

Antitumor Effect against Mouse HCC by Immunization with Tumor Lysate-pulsed DCs and Systemic Administration of IL-12. Next we examined whether systemic administration of IL-12 enhances the therapeutic potential of immunization with tumor lysate-pulsed DCs in this mouse HCC tumor model. BALB/c mice were injected s.c. with 2 x 10^5 parental BNL cells. On day 14, the s.c. tumor size grew to ~70 mm^3. Mice were immunized with BNL lysate-pulsed DCs plus IL-12, BNL lysate-pulsed DCs alone, IL-12 alone, or PBS. As shown in Fig. 2A, tumor rejection was observed in three of eight mice treated with BNL lysate-pulsed DCs plus IL-12, and the growth of BNL tumors in the remaining five mice was significantly inhibited compared with the mean size of tumors in control groups.

Statistical Analysis. Statistical significance of differences between the two groups was determined by applying Student’s t test or two-sample t test with Welch correction after each group had been tested for equal variance. Fisher’s exact probability test. Statistical significance of the differences in three groups was determined by applying one-way ANOVA. We defined statistical significance as P < 0.01.

PRODUCTION.

Cytolytic assay. Recombinant murine IL-12 was generously provided by Dr. Bich Nguyen (Genetics Institute, Cambridge, MA). BALB/c mice received s.c. injection of 1 x 10^6 BNL tumor lysate-pulsed DCs at 7-day intervals over a 3-week period. After dilution in 0.1% mouse albumin carrier protein, murine IL-12 was given i.p. for 5 consecutive days after each immunization with BNL tumor lysate-pulsed DCs. Control groups of mice received either BNL tumor lysate-pulsed DCs alone, IL-12 alone, or no treatment (PBS). Cytolytic assay was performed approximately 7 days after the final immunization. The spleen cells from immunized mice (3 x 10^6 cells/well) were cocultured with MMC-treated BNL cells (3:1 responder:tumor cell ratio) in 2 ml of complete T-cell medium (RPMI 1640 containing 50 μM 2-mercaptoethanol) in 24-well tissue culture plates at 37° C in a humidified atmosphere containing 5% CO₂. One day after culture initiation, mouse IL-2 (Genzyme, Cambridge, MA, USA) was added to the cultures to yield final concentrations of 30 units/ml. At 3 and 6 days of culture, 1 ml of culture medium was removed and replaced with 1 ml of fresh complete medium containing IL-2 (30 units/ml). Cytotoxic and cytokine production was assessed using a gamma counter. Supernatants were collected and stored at -70° C in aliquots until use. DCs were pulsed with tumor lysates (100 g/mouse/day) was given i.p. for 5 consecutive days after each immunization with BNL tumor lysate-pulsed DCs. Control groups of mice received either BNL tumor lysate-pulsed DCs alone, IL-12 alone, or PBS. Tumor size was determined as described above.
in the mice treated with the other protocols \((P < 0.01\) at 3, 4, 5, 6, and 7 weeks \(\text{versus PBS and versus IL-12 alone; } P < 0.01\) at 5, 6, and 7 weeks \(\text{versus BNL lysate-pulsed DCs alone})\). Although immunization with BNL lysate-pulsed DCs alone did not lead to complete regression of established tumors, tumor growth was significantly inhibited compared with that seen in mice treated with IL-12 or PBS \((P < 0.01\) at 3, 4, 5, 6, and 7 weeks \(\text{versus PBS and versus IL-12 alone})\). In contrast, tumor growth was not inhibited in mice that received IL-12 alone. These results demonstrated that IL-12 administration enhanced the therapeutic effect of BNL lysate-pulsed DCs against a pre-established BNL tumor. We further examined the specificity of this combination therapy. BALB/c mice were immunized with BNL lysate-pulsed DCs plus IL-12 or PBS after receiving a s.c. injection of Colon26 cells. As shown in Fig. 2A, immunization of mice with BNL lysate-pulsed DCs plus IL-12 had no effect on the growth of the unrelated Colon26 colon tumor.

**Requirement of Both CD8\(^+\) and CD4\(^+\) T Cells, but not NK Cells, for Antitumor Effect of Immunization with Tumor Lysate-pulsed DCs and Administration of IL-12.** To identify the population of T cells and NK cells required for the induction of the antitumor activity, mice were depleted of CD4\(^+\) or CD8\(^+\)-cell subsets or NK cells before the immunization. As shown in Fig. 3, tumor growth of BNL cells in CD8\(^-\)-T-cell-depleted mice was completely restored at the levels of PBS-injected mice, and tumor growth of BNL cells in CD4\(^+\)-T-cell-depleted mice was partially restored (Fig. 3, A and B). In contrast, tumor growth in NK cell-depleted mice was not restored at all (Fig. 3C). These data indicated that CD8\(^+\) T cells are essential for inducing the antitumor effect conferred by tumor lysate-pulsed DCs and IL-12 and that CD4\(^+\) T cells also have a role in this.

**DISCUSSION**

HCC recurrence may develop, at least in part, from residual tumor existing at the time of the initial therapy. In this study, we investigated...
therapeutic potential of immunization with BNL tumor lysate-pulsed DCs combined with administration of IL-12 against a preexisting BNL tumor. Immunization with BNL tumor lysate-pulsed DCs prevented the growth of BNL tumor in syngeneic mice, whereas administration of IL-12 did not show an antitumor effect on BNL cells.

When injected at the time of DC immunization, IL-12 enhanced the antitumor effect of DC immunization, as evidenced by significant suppression of tumor growth compared with DC immunization alone. These results suggest that the combination of IL-12 and tumor lysate-pulsed DCs may be a promising approach to suppress the growth of residual HCC after initial therapy.

We also demonstrated here that immunization with BNL tumor lysate-pulsed DCs induced BNL-specific cytolytic activity and IFN-γ production of lymphocytes in syngeneic mice. More importantly, administration of IL-12 at the time of immunization clearly enhanced these responses. Zitvogel et al. (26) reported that the antitumor effect of DC-based vaccination is dependent on production of Th1-associated cytokines such as IFN-γ, tumor necrosis factor α, and IL-12. Therefore, IFN-γ production enhanced by IL-12 may play an important role in increased antitumor activity in vivo.

In this study, IL-12 itself did not induce an immune response or an antitumor effect against BNL cells. Therefore, IL-12 may serve to up-regulate the effect of DC-based vaccination in vivo. Kelleher et al. (23) reported that administration of IL-12 increases the stimulatory capacity of bone marrow-derived DCs in vitro. In addition, Sato et al. (27) recently reported that Th1-biasing cytokines such as IL-12, in addition to their effect on Th cell differentiation, may play a critical role in the functional skewing of DCs. Our finding that administration of IL-12 at the time of DC immunization enhanced IFN-γ production of lymphocytes is consistent with the idea that IL-12 up-regulates the Th1-type immune response initiated by DC immunization. It has also been shown that IL-12 enhances NK and CTL activities (18–20) and directs a cell-mediated immune response by generating a Th1-type immune response and inhibiting differentiation of Th2-type lymphocytes (21, 22). We did not directly prove that CD8+ or CD4+ T cells accumulate in the tumor and function as effector cells for killing tumor cells. However, our in vivo lymphocyte depletion experiments demonstrated that CD8+ T cells and CD4+ T cells, but not NK cells, are required for the generation of anti-BNL immunity conferred by BNL lysate-pulsed DCs and IL-12. Taken together, we speculate that systemic administration of IL-12 may enhance the antigen-presenting function of DCs toward CD8+ T cells and activation of CD4+ T cells, particularly their Th-1 subsets, thereby increasing the in vivo effect of DC-based immunization.

The tumor growth of Colon26 cells, which is syngeneic to BALB/c mice, was not inhibited by this combination of BNL lysate-pulsed DC vaccine and IL-12. These results suggested that this combination induced BNL tumor-specific antitumor immunity in vivo. The use of tumor lysate as the source of antigens offers the advantage of potentially providing the DCs with multiple tumor-associated antigens in the form of both helper- or CTL-defined epitopes for presentation to T cells, which could overcome tumor evasion by stimulating both arms of cellular immune response. One potential disadvantage of this approach is the possible induction of an autoimmune response to self or to normal tissue antigens present in the tumor lysate. To examine whether immunization of tumor lysate-pulsed DCs and/or administration of IL-12 cause hepatocyte toxicity, mice treated with IL-12, BNL lysate-pulsed DCs or IL-12 in combination with lysate-pulsed DCs were sacrificed at 1 week after the end of the treatment. Under this experimental condition, we did not find any histological findings, such as hepatocyte apoptosis, infiltration of inflammatory cells, and fibrosis in the liver, or increases in serum alanine aminotransferase levels. Vierboom et al. (28) also reported that the expression of self-antigens on tumor cells could serve as a target for specific CTLs without any demonstrable damage to normal tissue. However, because
HCC frequently occurs in patients with impaired hepatic reserve, this issue should be carefully examined when DC-based immunotherapy is applied for human HCC treatment.

Despite recent progress in HCC therapy, there are HCCs that cannot be cured by conventional treatment. The need is great for innovative therapeutic methods. We have shown here that administration of IL-12 could enhance the therapeutic antitumor effect of immunization with tumor lysate-pulsed DCs in a mouse HCC tumor model. These findings raise the possibility that administration of IL-12 and immunization with tumor lysate-pulsed autologous DCs may have therapeutic potential for human HCC treatment.

ACKNOWLEDGMENTS

We thank Dr. Bich Nguyen for providing recombinant mouse IL-12.

REFERENCES


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