Enhanced Induction of Antitumor T-Cell Responses by Cytotoxic T Lymphocyte-associated Molecule-4 Blockade: The Effect Is Manifested Only at the Restricted Tumor-bearing Stages

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ABSTRACT

Cytotoxic T lymphocyte-associated molecule-4 (CTLA-4), a second counterreceptor for the B7 family of costimulatory molecules, functions as a negative regulator of T-cell activation. Here, we investigated whether the blockade of the CTLA-4 function leads to enhancement of antitumor T-cell responses at various stages of tumor growth. Unfractionated spleen cells taken from CSA1M fibrosarcoma-bearing mice 1–2 weeks after CSA1M cell implantation (early tumor-bearing mice) contained tumor-primed T cells that produced interleukin 2 and IFN-γ through collaboration with antigen-presenting cell-binding tumor antigens when cultured in vitro. However, this initial lymphokine-producing capacity decreased at later stages of tumor growth (7–10 weeks after tumor cell implantation). Anti-CTLA-4 monoclonal antibody (mAb) was added to whole-spleen cell cultures from early or late tumor-bearing mice. Spleen cells from early tumor-bearing mice exhibited enhanced production of interleukin 2 and IFN-γ upon in vitro culture in the presence of anti-CTLA-4 mAb. However, addition of anti-CTLA-4 mAb to whole-spleen cell cultures from late tumor-bearing mice failed to display such an enhancement. Consistent with these in vitro results, the in vivo antitumor effect of anti-CTLA-4 administration was observed in a tumor-bearing stage-restricted manner; in vivo administration of anti-CTLA-4 (1 mg/mouse, three times at 1-week intervals) into early tumor-bearing mice resulted in regression of growing tumors, whereas the same treatment did not affect tumor growth when performed for late tumor-bearing mice. Similar anti-CTLA-4 effect was observed in another tumor (OV-HM ovarian carcinoma) model. These in vitro and in vivo results indicate that CTLA-4 blockade in tumor-bearing individuals enhances the capacity to generate antitumor T-cell responses, but the expression of such an enhancing effect is restricted to early stages of tumor growth.

INTRODUCTION

It has been established in recent years that tumor cells express tumor antigens that are recognizable by antitumor T cells (1, 2). However, antitumor cell responses leading to tumor rejection cannot be developed, even in most experimentally induced murine tumor models. One reason for this unsuccessful rejection would be that various mechanisms of immunosuppression are generated in the tumor-bearing state, either by tumor cells or host lymphoid cells (reviewed in Ref. 3). It may also be that a T-cell response to weakly immunogenic tumor antigens is modulated when such a response is chronically perpetuated.

T-cell activation requires the delivery of at least two signals by APCs: (i) via a T-cell receptor through recognition of an antigen-MHC complex and (ii) via a second costimulatory receptor. CD28 is the best characterized costimulatory receptor that is constitutively expressed on mature T cells (3). Upon stimulation with the B7 family of molecules on APC, CD28 appears to act as the principal costimulatory receptor (6, 7). Following activation, T cells also express CTLA-4 (8), a second receptor for B7 family members that binds these members with a much higher affinity (6, 9, 10). Compelling evidence suggests, however, that CTLA-4 might act as a negative regulator of T-cell activation rather than as a positive costimulatory receptor (11–13). Cross-linking of CTLA-4 molecules on the T-cell surface results in decreased T-cell proliferation and IL-2 production (11, 13). Moreover, in vivo blockade of CTLA-4 interactions with anti-CTLA-4 mAb was observed to induce rejection of implanted tumor cells. However, it was not examined whether antitumor T-cell responses were enhanced by CTLA-4 blockade. It is possible that, although antitumor T cells are once activated in tumor-bearing individuals, their activation is reduced by CTLA-4 expressed on activated T cells, and if so, CTLA-4 blockade leads to enhancement of antitumor T-cell responsiveness.

Here, we investigated the effect of CTLA-4 blockade on antitumor T-cell responses at various stages of tumor growth. The results show that CTLA-4 blockade at early stages of tumor growth results in enhanced induction of antitumor T-cell responses along with regression of established tumors. However, the same treatment failed to induce enhancement of in vitro and in vivo T-cell responses at late tumor-bearing stages. The results suggest that CTLA-4 blockade has the potential to enhance antitumor T-cell responses, but this effect is manifested only at early stages of tumor growth.

MATERIALS AND METHODS

Mice. Male BALB/c and female (C57BL/6 × C3H/He)F1 mice were obtained from Shizuoka Experimental Animal Center (Hamamatsu, Japan) and used at 6–9 weeks of age.

Tumor Cell Lines. The following two tumor cell lines were used: CSA1M fibrosarcoma (14) and OV-HM ovarian carcinoma (15) were kindly provided by Dr. Takato O. Yoshida (Hamamatsu University School of Medicine, Hamamatsu, Japan) and Dr. Ohtsura Niwa (Hiroshima University, Hiroshima, Japan). These cloned tumor cell lines were maintained in RPMI 1640 supplemented with 10% FCS at 37°C in a humidified atmosphere with 5% CO2.

mAb. Hybridoma cells producing anti-CTLA-4 mAb (Ref. 16; UC10-4F10-11) were established in one of our laboratories (J. B.). MAb was purified from ascitic fluid of hybridoma cells. Hamster control immunoglobulin was purchased from Cappel Research Products (Cochraneville, PA).

Preparation of Lymphokine Sample (Whole-Spleen Cell Culture System). Unfractionated spleen cells (5 × 106/ml) from tumor-bearing mice were cultured without addition of exogenous tumor antigens in 24-well culture
plates (Corning 25820; Corning Glass Works, Corning, NY) in a volume of 2 ml of RPMI 1640 supplemented with 10% FCS (17). After incubation at 37°C in a humidified incubator (5% CO2) for 48 h, culture supernatant was harvested by centrifugation and stored at −20°C until use.

Assay Systems for Lymphokine Activity/Concentration. Supernatants were assayed for IL-2 activity according to their ability to support the proliferation of the IL-2-dependent T-cell line, CTLL-2, as described (17). The absolute concentrations of IL-2 were determined by extrapolation from the standard curve that was generated by using known amounts of recombinant IL-2. The concentration of IFN-γ was measured using ELISA kits obtained from Genzyme (Cambridge, MA).

RESULTS

Our previous study (18) demonstrated that unfractionated spleen cells from CSA1M tumor-bearing mice contain tumor-sensitized T cells and APC-binding CSA1M antigens in vivo. During in vitro cultures of this whole-spleen cell population, various lymphokines/cytokines are produced by such T cells in response to APC-presenting CSA1M antigens (whole-spleen cell culture system). We examined the capacity of spleen cells from various stages of CSA1M or OV-HM tumor-bearing mice to produce IL-2 and IFN-γ in the whole-spleen cell culture system. Spleen cells from early, intermediate, or late stages of tumor-bearing mice were cultured for 48 h without addition of exogenous tumor antigens. Culture supernatants were collected and tested for IL-2 activity and IFN-γ concentration. The results of Fig. 1 show that spleen cells from either CSA1M or OV-HM tumor-bearing mice at early stages produced higher levels of IL-2 and IFN-γ than those generated by normal spleen cells. However, this cytokine production decreased with the progress of tumor-bearing stages in both tumor models.

Before we investigated the effect of anti-CTLA-4 mAb on antitumor T-cell responses, we examined the efficacy of the anti-CTLA-4 mAb that we prepared. This was done by confirming its enhancing effect on an allogeneic MLR that was reported in an initial study (16). Under conditions in which stimulation with a low concentration of allogeneic cells generates only a slight level of T-cell proliferation, addition of anti-CTLA-4 mAb at a high dose (20 μg/ml) resulted in strikingly enhanced MLR (Fig. 2). Either anti-CTLA-4 or control Ab was not mitogenic because the Ab itself did not stimulate the proliferation of responding cells alone (data not shown).

Various doses of anti-CTLA-4 mAb were added to whole-spleen cell cultures from normal mice or early or late stages of tumor-bearing mice (Figs. 3 and 4). Similar patterns of lymphokine-producing capacities to those in Fig. 1 were again seen in cultures containing control hamster IgG. Addition of anti-CTLA-4 mAb to cultures from early tumor-bearing mice resulted in enhanced production of IL-2 and IFN-γ compared to the production in cultures containing control IgG. This was observed in both CSA1M and OV-HM tumor models. However, the same anti-CTLA-4 treatment did not induce enhancement of IL-2 and IFN-γ production by spleen cells from late tumor-bearing mice.

We next investigated whether anti-CTLA-4 mAb, when administered into various stages of tumor-bearing mice, affects tumor growth. Anti-CTLA-4 mAb was given at a dose of 1 mg/mouse, three times at 1-week intervals, in the CSA1M tumor model. When anti-CTLA-4 treatment was started at early stages of CSA1M.
Fig. 3. Enhanced IL-2 production by spleen cells from early tumor-bearing mice in the presence of anti-CTLA-4 mAb. Unfractionated spleen cells from normal mice or early or late stages of CSA1M (A) or OV-HM (B) tumor-bearing mice (three mice/group) were cultured for 48 h in the presence of anti-CTLA-4 mAb or control hamster IgG at the indicated concentrations. Culture supernatants were assessed for IL-2 activity.

Tumor growth, i.e., on day 10 (Fig. 5A) or on day 14, tumor regression was induced in all or more than half of the animals. Control hamster immunoglobulin did not produce any significant protective effect (data not shown). However, the same protocol of treatment starting on day 28 (Fig. 5D) or at later stages (data not shown) failed to induce antitumor effects. The mice whose tumors regressed by anti-CTLA-4 treatment were rechallenged with CSA1M tumor cells. All animals rejected a second challenge (Fig.

Fig. 4. Enhanced IFN-γ production by spleen cells from early tumor-bearing mice in the presence of anti-CTLA-4 mAb. Portions of the same culture supernatants as those used in Fig. 3 were assessed for IFN-γ concentration.
Fig. 5. Effect of anti-CTLA-4 treatment on CSA1M tumor growth. Anti-CTLA-4 mAb was i.p. injected into CSA1M tumor-bearing mice at a dose of 1 mg/mouse three times at 1-week intervals. Anti-CTLA-4 treatment was started 10 (A), 14 (B), 21 (C), and 28 (D) days after implantation with 3 × 10⁶ CSA1M cells.

6), indicating that anti-CTLA-4 induced tumor regression is associated with the acquisition of antitumor immune responses.

Similar in vivo experiments were performed in the OV-HM tumor model (Fig. 7). In this model, in which tumor growth was fast compared to that in the CSA1M model, tumor regression was induced only when anti-CTLA-4 treatment was started simultaneously with tumor implantation. Almost no protective effect was induced, even by anti-CTLA-4 injections starting as early as 14 days after tumor implantation.

DISCUSSION

Our present results show that CTLA-4 blockade during interactions between T cells and APC from early tumor-bearing mice leads to enhanced lymphokine production by T cells. However, similar blockade failed to induce the restoration/enhancement of lymphokine production that is suppressed at late stages of tumor growth. Consistent with these in vitro results, in vivo CTLA-4 blockade by injections of anti-CTLA-4 mAb resulted in tumor regression/tumor growth inhibition only at early tumor-bearing stages. Thus, the present study indicates that CTLA-4 blockade is capable of enhancing antitumor T-cell responses in a tumor-bearing stage-restricted way.

An earlier study from our laboratory indicated that APCs from CSA1M tumor-bearing mice constitutively present processed tumor antigens in vivo (17). This was demonstrated by the fact that these APCs, when inoculated into normal recipient mice, produced tumor-specific protective immunity (17). We have further shown that antitumor T cells are primed in vivo with these APCs and that antitumor responses (lymphokine production) are generated through collaboration between tumor-primed T cells and tumor APC from early tumor-bearing mice when cultured, even without the addition of exogenous tumor antigens (whole-spleen cell culture system; Ref. 18).

Here, we have found an enhancing effect of anti-CTLA-4 mAb on the function of antitumor T cells from early tumor-bearing mice in the whole-spleen cell culture system. This anti-CTLA-4 effect may be explained by considering differential function between CD28 and CTLA-4 molecules. CD28 is expressed constitutively on essentially all CD4⁺ and most CD8⁺ mature T cells. In contrast, CTLA-4 is not expressed on resting T cells but is induced after the initial step of T-cell activation. Although the function of CD28 (6, 7) has been well investigated, the biological significance of the coreceptor of the B7 family, the CTLA-4 molecule (8), has long been unclear. Recent
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Fig. 7. Effect of anti-CTLA-4 treatment on OV-HM tumor growth. Anti-CTLA-4 mAb was given to OV-HM tumor-bearing mice in the same protocol as conducted in Fig. 5. Anti-CTLA-4 treatment was started 0 (A), 3 (B), 7 (C), or 14 (D) days after implantation with $2 \times 10^5$ OV-HM cells.

reports have, however, suggested that CTLA-4 transmits a negative signal to T cells (11–13); cross-linking CTLA-4 with the anti-CTLA-4 mAb inhibits T-cell proliferation. Moreover, because CTLA-4 has a greater affinity for B7 than CD28 molecules, CTLA-4 molecules may also interfere with the interaction of CD28 and B7 by competing for B7 binding. Therefore, it may be possible that persistent stimulation with tumor antigens plus APCs induces CTLA-4 expression on an antitumor T-cell population and that CTLA-4 negatively regulates the responses of antitumor T cells by interfering with the CD28 costimulatory pathway. In fact, CTLA-4 blockade by anti-CTLA-4 mAb induced enhanced antitumor T-cell responsiveness. Namely, addition of anti-CTLA-4 mAb to whole spleen cell cultures from early tumor-bearing mice resulted in enhanced levels of lymphokines production by antitumor T cells. These observations are consistent with the results that allogeneic MLR is augmented by CTLA-4 blockade (Ref. 11 and Fig. 1).

The capacity of antitumor T cells to produce IL-2/IFN-γ decreased at late stages of tumor growth (18). Our previous study (19) has shown that culture of spleen cells from early stages of tumor-bearing mice generates Th2 activity, as exemplified by the production of IL-4. However, as the capacity to produce Th1 cytokines decreased, the production of Th2 cytokines decreased with the progress of tumor-bearing stages (19). Therefore, it is unlikely that the suppression of IL-2/IFN-γ production at late stages of tumor growth is due to Th2-derived cytokine-mediated regulation. Addition of anti-CTLA-4 mAb to cultures of antitumor T cells at these stages failed to enhance/restore their responsiveness. Differential restoration of in vitro antitumor T-cell responsiveness by anti-CTLA-4 at early versus late stages correlated with the capacity of anti-CTLA-4 to modulate tumor growth in vivo. Three injections of anti-CTLA-4 mAb to early stages (10–14 days after CSA1M cell implantation) of CSA1M-bearing mice induced high frequencies of complete tumor regression. Because anti-CTLA-4 mAb itself did not react with CSA1M tumor cells, it appears that anti-CTLA-4-mediated tumor inhibition is induced, based on an enhancement of antitumor responses rather than the direct effect of anti-CTLA-4 on tumor cells. Moreover, this regression was found to be associated with the acquisition of antitumor protective immunity. In contrast, anti-CTLA-4 mAb produced almost no protective immunity when administered at late stages. These results may suggest that antitumor T cells have been deleted or have received an irreversible effect at late stages of tumor growth. However, the following observation may make this possibility unlikely: administration of IL-12 into late stages of CSA1M or OV-HM-bearing mice resulted in complete tumor regression through mechanisms involving the reversal of suppressed IFN-γ production by antitumor T cells and the establishment of a tumor-specific immune response (20, 21).

A number of studies have indicated that immune dysfunction in a generalized or an antitumor T-cell response is induced in the tumor-bearing state, especially at late stages of tumor growth (reviewed in Ref. 3). For example, high levels of transforming growth factor-β and IL-6 are detected in the circulation from late stages of tumor-bearing individuals (19, 22). Because these cytokines function to suppress the generation of cytotoxic CTL responses (23) and the production of antitumor effector cytokines, such as IFN-γ (19, 24), enhanced production of transforming growth factor-β and IL-6 could represent an immunosuppressive mechanism at late tumor-bearing stages. Although CTLA-4 blockade has potential to enhance antitumor T-cell responses, such manifestation could be influenced by additional immunoregulatory mechanisms generated, depending on the tumor-bearing stages.

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