Enhanced Induction of Very Late Antigen 4/Lymphocyte Function-associated Antigen 1-dependent T-Cell Migration to Tumor Sites following Administration of Interleukin 12

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ABSTRACT

Administration of interleukin 12 (IL-12) into mice bearing CSA1M, OV-HM, Meth A, or MCH-1-A1 tumor induced complete regression of CSA1M and OV-HM tumors but induced only a slight growth inhibition of Meth A and MCH-1-A1 tumors. These effects of IL-12 were associated with high and only marginal levels of T-cell infiltration into CSA1M/OV-HM and Meth A/MCH-1-A1 tumor masses, respectively. Here, we investigated the role of IL-12 in the induction of T-cell migration. Spleen cells from untreated or IL-12-treated CSA1M-bearing mice were stained in vitro with a fluorescent chemical and transferred i.v. into IL-12-untreated CSA1M-bearing mice. Migration of donor cells was quantitated by counting the number of fluorescent cells on cryostat sections of tumor masses. Although only a slight migration was detected for spleen cells from IL-12-untreated CSA1M-bearing as well as IL-12-treated or untreated MCH-1-A1 tumor masses, enhanced migration was observed for cells from IL-12-treated CSA1M-bearing mice. A similar enhanced migration was observed for the OV-HM model. In contrast, such an enhancement was only marginal in the Meth A and MCH-1-A1 models. Immunohistochecmical studies of tumors from IL-12-treated mice revealed that the predominant T-cell subset was CD4+ in CSA1M and CD8+ in OV-HM tumor masses. Consistent with this observation, the dominant subset of migrating T cells was found to be CD4+ in the CSA1M and CD8+ in the OV-HM models. T-cell migration was inhibited by pretreatment of recipients with either combination of anti-very late antigen 4 + anti-vacular cell adhesion molecule 1 or anti-lymphocyte function-associated antigen 1 + anti-intercellular adhesion molecule 1 monoclonal antibody. These results indicate that IL-12 can confer T cells with a capacity to migrate to tumor sites through very late antigen 4/lymphocyte function-associated antigen 1 adhesion pathways and that the in vivo acquisition of such a capacity following IL-12 treatment correlates with the induction of tumor regression.

INTRODUCTION

IL-121 has been shown to induce a number of biological effects (1, 2) that are consistent with its potential role as an antitumor agent. This cytokine enhances NK (3) and CTL (4) activities; acts as an NK and T-cell growth factor (5–7); stimulates secretion of various cytokines, particularly IFN-γ, by NK and T cells (3, 8); and promotes maturation of the Th1 helper cell subset (1, 9). In addition, IL-12 induces lymphoid cells to express a number of surface molecules including IL-2 receptor (10) and adhesion molecules (11, 12). Each of these biological properties has been considered to contribute to controlling tumor growth.

Materials and Methods

Tumor Cell Lines. The following four tumor cell lines were used: CSA1M fibrosarcoma (18), OV-HM ovarian carcinoma (19), Meth A fibrosarcoma, and MCH-1-A1 fibrosarcoma (20). CSA1M and OV-HM tumors were kindly provided by Dr. Takato O. Yosida (Hamamatsu University School of Medicine, Hamamatsu, Japan) and Dr. Ohtsura Niwa (Hirosima University, Hirosima, Japan). CSA1M fibrosarcoma was induced in a male BALB/c mouse with the Schmidt-Ruppin strain of Rous sarcoma virus and shown to produce no sarcoma virus (18). An ovarian tumor, OV2944, was induced in a female (C57BL/6 × C3H/He)F1 mouse (hereafter called B6C3F1) by giving a single whole-body neutron irradiation, and a cloned line (designated OV-HM) was isolated from the parental line (19). MCH-1-A1 was a fibrosarcoma cell clone obtained from the parental line which was induced in a female C3H/HeJ mouse with methylcholanthrene in our laboratory (20). These tumor cell lines were maintained in RPMI 1640 supplemented with 10% FCS at 37°C in a humidified atmosphere with 5% CO2, and Meth A tumor was maintained by i.p. passages in syngeneic BALB/c mice.

Mice. Male BALB/c, female C3H/HeJ, and female B6C3F1 mice were obtained from Shizuoka Experimental Animal Center (Hamamatsu, Japan) and used at 6–9 weeks of age.
Preparation of Tumor-bearing Mice. Mice were inoculated s.c. with CSA1M (3 × 10^7/mouse), OV-HM (5 × 10^7/mouse), Meth A (5 × 10^7/mouse), or MCH-1-A1 (5 × 10^7/mouse) tumor cells.

Reagents. Murine IL-12 was kindly provided by Dr. B. Hubbard (Genetics Institute, Cambridge, MA). It was purified from the supernatants of Chinese hamster ovary cells transfected with the p35 and p40 cDNA plasmids. A fluorescent dye, PKH-26-GL (abbreviated as PKH-26), was purchased from Sigma Chemical Co. (St. Louis, MO). Anti-LFA-1 and anti-ICAM-1 hybridomas were purchased from American Type Culture Collection (American Type Culture Collection, Rockville, MD). Anti-VLA-4 and anti-VCAM-1 hybridomas were kindly provided by Dr. Kensuke Miyake (Saga Medical College, Saga, Japan). mAbs were prepared from ascitic fluid of each hybridoma.

Labeling of Cells with a Fluorescent Dye. Staining was performed according to the manufacturer's recommended procedure. Briefly, spleen cells suspended to a concentration of 5 × 10^7/ml in 1 ml of diluent were allowed to react with 5 × 10^-6 M PKH-26 solved in 1 ml of diluent for 5 min at 37°C. Labeling was stopped by adding 2 ml of FCS, and cells were washed five times with RPMI 1640 containing 10% FCS.

A Lymphoid Cell Migration Assay. The assay system was essentially the same as described previously (21). Mice with similar tumor sizes (approximately 7 mm in diameter) were used as recipients for this assay. PKH-26-labeled spleen cells (1–10 × 10^7 cells in 1 ml of RPMI 1640) were injected i.v. into recipient (IL-12-untreated homologous tumor-bearing) mice. At various time points after injection, tumor masses were removed and cryostat sections were prepared. The entry of fluorescence-labeled donor cells was quantitated under a fluorescence microscope and expressed as the mean cell number ± SE per section.

Treatment of Spleen Cells with Anti-CD4 and/or Anti-CD8 plus Complement. Treatment in vitro of spleen cells with anti-CD4 and/or anti-CD8 mAb plus complement was performed as described (22).

Histological Examination. Tumor masses were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with H&E for histological examination.

Staining Procedure of Immunohistochemical Examination. The following reagents were purchased to perform immunohistochemical examination: biotinylated rat antimouse CD4 and anti-CD8 mAbs were from PharMingen (San Diego, CA) and VectaStain Elite ABC kit was from Funakoshi (Tokyo, Japan). Cryostat sections (6 μm) were cut, air dried, fixed in acetone at room temperature for 2–3 min, and then washed five times with PBS/0.05% Tween 20 at 4°C for 2 min, and the VectaStain ABC solution was added and incubated at room temperature for 30 min. After a final wash, the labeling was visualized with 0.03% DAB (Wako Pure Chemicals, Osaka, Japan) containing 0.1% hydrogen peroxide for several minutes.

RESULTS

Correlation of Tumor Regression with Massive Intratumoral Infiltration of CD4 and CD8 T Cells. Earlier studies from our laboratory have shown that administration of recombinant IL-12 to tumor-bearing mice induces various efficacies of antitumor effects depending on the tumor model (14, 17). To confirm this, IL-12 was injected i.p. into CSA1M- (Fig. 1A), OV-HM- (Fig. 1B), and Meth A- (Fig. 1C) bearing syngeneic mice. The dose of IL-12 was 0.5 μg/mouse/time five times every other day (Fig. 1). The results show that five injections of IL-12 resulted in complete tumor regression in the CSA1M as well as in OV-HM tumor models. In contrast, the same protocol of treatment failed to induce regression of Meth A tumors. Tumor regression was not induced in the MCH-1-A1 model as described in our earlier study (17). Previously, light microscopic examination revealed only marginal cellular infiltration in either type of tumor mass from IL-12-untreated mice (17). However, a massive infiltration of mononuclear cells was seen in both CSA1M and OV-HM tumor masses obtained from IL-12-treated mice, whereas only slight cellular infiltration was observed in the Meth A^4 and MCH-1-A1 tumor mass (17).

To determine whether mononuclear cells detected in the regressing CSA1M and OV-HM tumor masses represent T cells and, if so, which subset (CD4 or CD8) is predominant, immunohistochemical examination was performed. A large number of CD4^+ and CD8^+ T cells were stained in cryostat sections of both CSA1M- and OV-HM-regressing tumors (Fig. 2). However, there was a difference in the dominance of infiltrating CD4^+ and CD8^+ T cells between the two tumor models: in a regressing CSA1M tumor mass, a large number of CD4^+ T cells and a small number of CD8^+ T cells were present at the outer area of the tumor mass (Fig.

Unpublished observations.
2, left panels), although a moderate number of CD8+ T cells also migrated into the inner area (14). Overall, CD4+ T cells dominated in the CSA1M section. In contrast, the CD8+ was the predominant T-cell subset throughout the OV-HM section (Fig. 2, right panels). Mac-1+ cells, probably constituting macrophage and neutrophil populations (17), were also observed, whereas only a few NK1.1+ cells were detected (data not shown). These results indicate that the induction of tumor regression correlates with massive T-cell infiltration with either CD4 or CD8 dominance depending on the tumor model studied.

IL-12-induced Enhanced Migration of Spleen Cells from CSA1M or OV-HM Tumor-bearing Mice into Tumor Sites. Normal or tumor (CSA1M or OV-HM)-bearing mice were untreated or treated with IL-12. Spleen cells were obtained 2 days after the third IL-12 injection and labeled with PKH-26. The labeled spleen cells were transferred i.v. into homologous tumor-bearing mice not treated with IL-12. Twenty-four h later, the numbers of PKH-26-labeled donor cells were evaluated on the cryostat sections of tumor masses from recipient mice (Fig. 3). The results show that donor spleen cells from IL-12-treated or untreated normal mice or IL-12-untreated tumor-bearing mice all gave a low level of migration into the tumor. In contrast, a strikingly enhanced migration was observed in both tumor models when spleen cells from IL-12-treated tumor-bearing mice were used as donor cells. A set of results in both models are also illustrated by micrographs (Fig. 4). The migration was found to be time dependent after the donor cell transfer and donor cell dose dependent (data not shown).

Differential Migration of Spleen Cells into Tumor Masses in Various Tumor Models. We compared the degree of IL-12-induced spleen cell migration among various tumor models. A representative of three experiments performed is shown in Fig. 5. The IL-12-induced enhancement of spleen cell migration was again observed in the
Fig. 4. Fluoromicroscopic examination of CSA1M and OV-HM tumor masses from mice receiving four groups of donor cells. Spleen cells were obtained from the following groups of mice: A, untreated normal; B, IL-12-treated normal; C, untreated tumor bearing; and D, IL-12-treated tumor bearing.

induced by spleen cells from IL-12-untreated tumor-bearing mice. Treatment of spleen cells with either anti-CD4 or anti-CD8 mAb in the presence of complement induced different patterns of migration in the two tumor models: elimination of the CD4+ T-cell subset markedly decreased the number of migrating cells in the CSA1M model, and, conversely, the migration was reduced by elimination of the CD8+ subset.

Fig. 5. Differential inducibility of IL-12-enhanced migration in various tumor models. Spleen cells (1 x 10^7/mouse) from untreated or IL-12-treated tumor-bearing mice (indicated) were transferred into IL-12-untreated homologous tumor-bearing mice. The results are representative of three similar experiments. Bars, SE.

CSA1M and OV-HM models, whereas no enhancement was induced in the Meth A or MCH-1-A1 model following treatment with IL-12. Thus, the degree of IL-12-induced migration evaluated by our migration assay correlates with the antitumor in vivo efficacy of IL-12.

Differential Dominance of the CD4 or CD8 T-Cell Subset in the Respective CSA1M or OV-HM Tumor Mass. Spleen cells from IL-12-treated CSA1M or OV-HM-tumor-bearing mice were treated with anti-CD4 and/or anti-CD8 mAb plus complement. The resultant populations were labeled with PKH-26. Each population was tested for migration by transfer into IL-12-untreated homologous tumor-bearing mice. The results are summarized in Fig. 6. Simultaneous elimination of CD4 and CD8 T cells from the donor cell inoculum resulted in almost complete inhibition of migration in both tumor models. The reduced migration levels were comparable to those
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CD8^+ T-cell subset in the OV-HM model. The dominance of the CD4 versus CD8 T-cell subsets observed in the migration assay coincided with that seen in the immunohistochemical analyses on cryostat sections of CSA1M and OV-HM tumor masses from IL-12-treated mice (Fig. 2).

Adhesion Molecules Involved in T-Cell Migration. We finally investigated whether the migration detected in the present assay involves interactions between adhesion molecules expressed on T cells and endothelial cells. To block two different interactions, two combinations of mAbs (anti-VLA-4 + anti-VCAM-1 and anti-LFA-1 + anti-ICAM-1) were injected i.p. prior to transfer of donor spleen cells. To compare the degree of contribution of each mAb combination, the dose of mAbs was titrated separately for the two sets of combinations. The results of Fig. 7 demonstrate that both combinations of mAb induced a dose-dependent inhibition of migration. However, the blocking of the VLA-4–VCAM-1 pathway appeared to induce more potent inhibition of migration than that of the LFA-1–ICAM-1 pathway when compared at higher doses of mAbs injected. Similar patterns of blocking with two combinations of mAbs were observed in the OV-HM tumor model (data not shown). These results indicate that both pathways of VLA-4–VCAM-1 and LFA-1–ICAM-1 are involved in the IL-12-induced enhanced migration of splenic T cells, although the contribution may be slightly higher in the former than in the latter.

DISCUSSION

The present results obtained in various tumor models showed that the induction of tumor regression correlated with T-cell infiltration at tumor sites in situ. This study developed a lymphoid cell migration assay and demonstrated that IL-12 can confer T cells with a capacity to migrate into tumor masses. Such a capacity was generated in two tumor models in which high levels of intratumoral T-cell infiltration and complete tumor regression were induced by systemic administration of IL-12. Moreover, IL-12-induced T-cell migration was inhibited by blocking interactions between VLA-4 and VCAM-1 or between LFA-1 and ICAM-1. In the other two tumor models, neither enhanced T-cell infiltration nor tumor regression was observed. Thus, our results indicate that IL-12 treatment provides T cells with the ability to migrate into tumor sites in a VLA-4/LFA-1-dependent manner and that the manifestation of this IL-12 effect is critical for inducing tumor regression.

The capacity of IL-12 to induce T-cell migration into tumor sites should be considered from several aspects. First, it may be important to consider how such a capacity mediates the antitumor effect and is associated with the apparent requirement for IFN-γ. Thus far, we assume that the antitumor effects of IL-12 are based on IFN-γ production; similar effects should be generated by the administration of recombinant IFN-γ itself. However, previous studies (23, 24) have shown that administration of IFN-γ alone causes antitumor effects, but these are much less pronounced compared with the IL-12 effects (13–15). This discrepancy may be explained by the following possibility. Systemic administration of IFN-γ may fail to achieve adequate local concentration of this cytokine due to its short half-life (25). Effective IFN-γ levels necessary for tumor rejection could be achieved by tumor site-selective production of this cytokine in studies using tumor cells genetically engineered to produce IFN-γ (26, 27).

Effective IFN-γ levels necessary for tumor rejection could be achieved by tumor site-selective production of this cytokine in studies using tumor cells genetically engineered to produce IFN-γ (26, 27). Alternatively, the elevation of IFN-γ levels at tumor sites could be induced by intratumoral infiltration of lymphoid cells. Our previous work (14, 17) indicated that IL-12 administration in vivo induced not only IFN-γ production by T cells in tumor-bearing mice but also the accumulation of these T cells at tumor sites. The present study directly demonstrated the capacity of IL-12 to induce in vivo migration of IL-12-stimulated T cells to tumor sites. Moreover, our previous study (14) showed that even when IFN-γ mRNA expression in lymphoid organs such as spleens ceased after suspending IL-12 administration, tumor-infiltrating T cells expressed high levels of IFN-γ mRNA. Thus, the capacity of IL-12 for inducing T-cell migration could contribute to generating the mechanisms by which IFN-γ is produced in a tumor site-selective manner and functions efficiently to exert its antitumor effect.

Second, our present results demonstrated that the migration of IL-12-stimulated T cells to tumor sites depends on the interactions between VLA-4/LFA-1 and VCAM-1/ICAM-1 molecules. Recent studies have shown that IL-12 enhances the binding activities of VLA-4 and/or LFA-1 on NK or T cells with VCAM-1 and/or ICAM-1 molecules (11, 12). The involvement of these two interactive units in the recruitment of T cells to inflamed sites has been well established by the blocking experiments using mAbs against these adhesion molecules (28–33). In these previous studies, blocking each of the VLA-4–VCAM-1 and LFA-1–ICAM-1 pathways significantly reduced tissue/organ inflammation and prevented the manifestation of diseases. Likewise, in the present study, the migration of T cells from IL-12-treated tumor-bearing mice was found to be inhibited by blocking either set of adhesive interactions. This could be explained by the
hypothesis that these two sets of adhesion mechanisms mediate mutually different or sequential processes of T-cell migration to targeted tissues/organisms.

It is well known that the expression of endothelial cell adhesion molecules such as ICAM-1 and VCAM-1 is up-regulated at the inflammatory site by inflammatory cytokines like IFN-γ (33). We have previously found constitutive IFN-γ mRNA expression induced by inflamed infiltrating T cells within tumor masses from IL-12-treated but not from untreated tumor-bearing mice (14, 17). The tumor vasculature in such IL-12-treated mice are likely to express enhanced levels of endothelial cell adhesion molecules. However, it remained to be solved whether endothelial cells in tumors have expressed sufficient levels of adhesion molecules for attracting tumor-migrating T cells. In the present study, splenic T cells from IL-12-treated tumor-bearing mice were shown to be capable of migrating into the tumor mass of an IL-12-untreated tumor-bearing recipient. These results strongly suggest that tumor vasculature in the CSA1M and OV-HM models expresses adhesion molecules necessary for T-cell migration. This possibility would be supported by the recent study which showed expression of VCAM-1 and ICAM-1 in human tumor tissue (34). However, adoptive transfer of splenic T cells from IL-12-treated mice into untreated tumor-bearing mice, unlike IL-12 injection, failed to induce complete tumor regression, although significant growth inhibition was observed. It is possible that administration of IL-12 itself not only induces the activation of T cells but also functions to augment the acceptability of tumor masses for migrating cells, including enhanced induction of adhesion molecules and chemokines.

In relation to the inducibility of intratumoral endothelial cell adhesion molecules, it should be investigated why IL-12-stimulated T cells in Meth A- or MCH-1-A1-tumor-bearing mice failed to migrate to tumor sites. We previously demonstrated that Meth A (35) and MCH-1-A1 (20) are both immunogenic tumors and that in vivo protective immunity can be easily induced. In the MCH-1-A1 model, IL-12 administration induces IFN-γ levels comparable to those observed in the CSA1M- and OV-HM-curable models, suggesting a similar level of T-cell activation. This suggests that the lack of enhanced intratumoral T-cell migration in the MCH-1-A1 model may be due to mechanisms other than the failure of IL-12 to activate T cells, including antitumor T cells. Additional studies will be required to investigate the expression of endothelial cell adhesion molecules in this noncurable model in comparison to that in the two other curable models. The necessity of such careful investigation would be strengthened by the data showing that the expression of the endothelial cell adhesion molecule is suppressed in some human tumors (36).

Another important point to be further addressed concerns the specificity of the tumor-migrating T cells. We found that T cells from IL-12-treated CSA1M (BALB/c origin)-bearing mice did not migrate into the Meth A tumor (BALB/c origin) mass and that T cells from IL-12-treated Meth A-bearing mice also failed to migrate into the CSA1M mass. However, these results do not necessarily establish the specificity of migrating T cells, because IL-12-induced enhancement of migration was not observed in the Meth A model. Therefore, the specificity of migrating T cells remains to be examined by developing another curable tumor model in the BALB/c strain and by using donor T cells prepared in two curable models.

Our results illustrate that in addition to IFN-γ production, IL-12 can promote the migration of T cells to tumor sites in a VLA-4/LFA-1-dependent way and that such T-cell migration correlates with the induction of tumor regression. It may be possible to investigate the failure of IL-12 to induce intratumoral T-cell migration by comparing tumor vasculature between curable and noncurable models. Such an investigation could also contribute to a better understanding of cellular and molecular mechanisms underlying IL-12-induced tumor regression as well as to attempting an approach to elicit T-cell migration even in noncurable tumor models.

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