Interleukin 12 Gene Therapy of MHC-negative Murine Melanoma Metastases

Patrizia Nanni, Ilaria Rossi, Carla De Giovanni, Lorena Landuzzi, Giordano Nicoletti, Antonella Stoppacciaro, Mariella Parenza, Mario P. Colombo, and Pier-Luigi Lollini


ABSTRACT

Immunological gene therapy of cancer relies heavily on the activation of T cells, but tumors with defects in MHC gene expression are not recognized by MHC-restricted T cells. To investigate the potential of cytokine genes for the therapy of MHC-negative tumors, we transduced B78H1, a class I-negative melanoma clone, with a polycistronic vector carrying murine interleukin (IL-12) genes. The clones produced studies 400–25,000 pg/ml IL-12; their in vitro growth properties were similar to those of parental cells. A complete inhibition of growth was observed in vivo both after s.c. and i.v. administration of all IL-12 clones. IL-12-transduced cells were also used as a therapeutic vaccine in mice bearing micrometastases by nontransduced parental cells. A significant (80–90%) reduction in the number of lung nodules was obtained. Immunohistochemical analysis and studies in immunocompromised hosts showed that T cells and natural killer cells had a significant role in the elimination of IL-12-releasing cells. In vivo hybridization with cytokine probes detected a strong increase in the proportion of leukocytes positive for IFN-γ, tumor necrosis factor α, IL-1β, and IFN-inducible protein 10 at the site of rejection of IL-12-engineered tumor cells. However, it was clear that the loss of in vivo growth was also due to T-cell- and natural killer cell-independent factors, possibly related to the angiogenic properties of IL-12. In conclusion, tumor therapy based on IL-12 gene transduction was effective on a MHC-negative metastatic tumor, suggesting a possible application to MHC-defective human neoplasms.

INTRODUCTION

The use of recombinant cytokines and of cells transduced with cytokine genes, in association with the cloning of specific tumor rejection antigens, has considerably increased the possibilities to induce or to boost an antitumor immune response based mainly on T lymphocytes. On the other hand, detailed studies of MHC gene products revealed that defects in class I MHC expression are widespread among human tumors. In fact, according to some estimates, most human tumors fail to express one or more class I MHC glycoproteins on their membrane (1, 2). A causal link between specific MHC class I loss and immunoselection by T lymphocytes has not yet been established; however, it is certain that the absence of a given MHC gene product prevents the recognition by T lymphocytes of the antigenic peptides for which the MHC product represents the restriction element (1).

The high prevalence of tumors with class I MHC defects indicates that specific strategies should be devised to cope with tumors that cannot be efficiently killed by MHC-restricted CTLs. A good candidate for this type of application is IL-12, which is able to activate both specific TH1 responses and nonrestricted NK effectors (3).

IL-12 is a heterodimeric cytokine produced by antigen-presenting cells, phagocytes, and granulocytes (3). A strong antitumor efficacy of rIL-12 or of cells engineered to produce IL-12 has been reported in a great variety of tumors (4–11). Antitumor activity of IL-12 is thought to be mediated mainly by in vivo induction of IFN-γ, given that the therapeutic efficacy of IL-12 is reduced in mice treated with anti-IFN-γ antibodies (5). The IFN-inducible chemokine IP10 has been indicated in turn as a further mediator of the antitumor activity of IL-12 (7).

In this paper, we show that IL-12 gene transduction of a MHC-negative melanoma clone results in a complete loss of malignancy and that IL-12-transduced cells can be used as a therapeutic vaccine against metastases produced by nontransduced MHC-negative parental cells.

MATERIALS AND METHODS

Cells. B78H1 is an amelanotic clone of B16 melanoma (12). B78H1 cells were cultured in DMEM (Life Technologies, Inc., Milan, Italy) supplemented with 10% fetal bovine serum (Life Technologies, Inc.).

Gene Transduction. IL-12-producing B78H1 cells were obtained by transduction with the polycistronic Lp400ip5SN retroviral vector coding for both IL-12 and the polyclonal antibody to the IL-12 receptor (IL-12R) CH11 (13). Cells were cultured in DMEM (Life Technologies, Inc., Milan, Italy) supplemented with 10% fetal bovine serum (Life Technologies, Inc.).

Mice. Eight-week-old C57BL/6NCrlBR (referred to as C57BL/6) male mice and 5-week-old nu/nu male mice on Swiss CD1 background were purchased from Charles River Laboratories (Calco, Italy) and treated according to European Community guidelines. To obtain NK-depressed animals, we injected some groups of mice i.v. with 0.4 ml of a 1:25 dilution of anti-asialo GM1, antiserum (WAKO, Dusseldorf, Germany) 24 h before cell injection. Some mice were treated every 3 days with anti-asialo GM1 at the concentration described above and with 100 µg of anti-IFN-γ mAb (AN18 hybridoma; kindly provided by Dr. G. Garotta, Roche, Basel, Switzerland).

Tumor Growth and Metastasis. Mice were challenged s.c. with 0.2 ml of a single-cell suspension containing 5 × 10⁵ (C57BL/6) or 10⁶ (nude mice) tumor cells; cell doses were chosen to obtain tumors with a similar incidence and growth curve in C57BL/6 and in nude mice. The incidence and the growth of tumors were evaluated twice weekly. Neoplastic masses were measured with calipers; tumor volume was calculated as m⁶ × [v/(a × b)]², in which a and b are two perpendicular major diameters. Experimental metastases were evaluated 28 days after the injection in a lateral tail vein of 5 × 10⁵ (C57BL/6), 10⁶ (nude mice), or 2.5 × 10⁶ (anti-asialo GM1-treated C57BL/6) tumor cells suspended in PBS. Lung nodules were contrasted with black India ink; all metastasis counts were performed on dissected lung lobes under a stereoscopic microscope.

Therapeutic Vaccination. Therapeutic vaccination started 1 day after B78H1 parental cell challenge and consisted of seven injections of 10⁶ viable or mitomycin C (80 µg/ml at 37°C for 45 min; Sigma)-treated cells 3–4 days apart.

Administration of Exogenous Mouse IL-12. Murine rIL-12, kindly provided by Dr. Maurice Gately (Hoffmann LaRoche, Nutley, NJ), was injected (0.1 µg/day diluted in PBS containing 100 µg/ml murine serum albumin) i.p. or at the site of s.c. vaccination. Starting 1 day after i.v. injection of tumor cells, mice received two courses of five daily i.p. injections with a 2-day...
Assays. Mixed lymphocyte tumor culture was performed in RPMI 1640 supplemented with 10% FCS (Hyclone, Logan, UT) using a modification of a previously reported procedure (18). Responders splenocytes were stimulated either with IL-2 (20 units) alone or with IL-2 and γ-irradiated (20,000 rad) class I-transfected B78H1 cells (12). Responders and stimulators were suspended to 2.5 × 10^5 and 2.5 × 10^4 cells/ml, respectively, and mixed in a total volume of 2 ml in 24-well plates (Costar, Cambridge, MA). Cultures were incubated in a humidified atmosphere of 5% CO₂ in air. The induction of a CTL response was assessed in standard 4-h ⁵¹Cr release assays as described previously (19). In CTL assays, B78H1 and class I transfected B78H1 cells were the specific targets; YAC-1 cells were used as controls for NK cell-mediated lysis.

RESULTS

IL-12 Gene Transduction in MHC-negative B78H1 Cells. B78H1 is a clone of B16 melanoma that does not express either class I or class II MHC gene products (Fig. 1; Ref. 12). IL-12 genes were transfected in B78H1 cells by means of a poly-cistronic retroviral vector. IL-12 released by the resulting clones ranged from 128 pg to 25 ng/ml/2 × 10^6 cells/48 h. Three clones named IL-12-400, IL-1216,000 and IL-1225,000 were chosen for further studies; the exponent in the name of each clone represents its IL-12 release in pg/ml.

In vitro growth properties of IL-12 clones, including cell morphology, growth rate, and MHC antigen expression, were similar to those of parental cells (data not shown).

IL-12 Release Abolishes B78H1 Tumorigenicity and Metastatic Ability. B78H1 cells and IL-12 clones were injected s.c. in immunocompetent C57BL/6 mice. B78H1 gave rise to rapidly growing tumors, but no tumor growth was ever observed in mice treated with IL-12-secreting cells (Fig. 2). These tumor-free mice, however, did not develop resistance to a subsequent challenge with B78H1 parental cells performed 60 days after the injection of IL-12 transfectants.

Similar results were obtained after i.v. injection of tumor cells: no metastases were ever observed in mice inoculated with high IL-12-secreting clones (Fig. 2).

The results indicate that IL-12 gene transduction abolished the in vivo tumorigenicity and metastatic potential of B78H1 melanoma cells.

Mixed Lymphocyte Tumor Culture and Cell-mediated Lympholysis Assays. Mixed lymphocyte tumor culture was performed in RPMI 1640 supplemented with 10% FCS (Hyclone, Logan, UT) using a modification of a previously reported procedure (18). Responders splenocytes were stimulated either with IL-2 (20 units) alone or with IL-2 and γ-irradiated (20,000 rad) class I-transfected B78H1 cells (12). Responders and stimulators were suspended to 2.5 × 10^5 and 2.5 × 10^4 cells/ml, respectively, and mixed in a total volume of 2 ml in 24-well plates (Costar, Cambridge, MA). Cultures were incubated in a humidified atmosphere of 5% CO₂ in air. The induction of a CTL response was assessed in standard 4-h ⁵¹Cr release assays as described previously (19). In CTL assays, B78H1 and class I transfected B78H1 cells were the specific targets; YAC-1 cells were used as controls for NK cell-mediated lysis.

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vivo growth of MHC-negative tumor cells. We then asked whether IL-12-transduced cells could be used as a therapeutic vaccine to inhibit the growth of nonengineered parental cells.

**Therapy of Lung Metastases.** Mice bearing B78H1 micrometastases were repeatedly vaccinated s.c. with live IL-12 clones. Both IL-12 clones, but not parental B78H1 cells, caused a significant (80–90%) reduction in the number of lung metastases (Table 1). No tumor was induced at the vaccination site; however, to perform a meaningful comparison with tumorigenic parental cells, vaccines were also pretreated in vitro with mitomycin C to block cell proliferation. Also, vaccination with mitomycin-treated IL-12 25'000 and IL-12 25'000 strongly inhibited the development of lung metastases (Table 1).

The efficacy of rIL-12 administration in the treatment of local or metastatic lesions was also evaluated. A 50% reduction in tumor volume was observed in mice bearing 7-day tumors of nonengineered B78H1 cells after two 5-day courses of rIL-12 given i.p. (data not shown). The antimetastatic efficacy of IL-12 gene-transduced cells was compared to the i.p. or s.c. administration of exogenous rIL-12. Both types of treatment seemed to reduce the metastatic load to a similar extent (Table 1).

**Tumor and Metastasis Growth in Immunocompromised Hosts.** To analyze the immune response involved in the rejection of IL-12 clones, we studied their growth in immunocompromised hosts (Fig. 3). The s.c. injection of IL-12 2000 in athymic nude mice gave rise to tumors with growth kinetics much slower than parental B78H1 tumors. Only a small proportion of nude mice receiving IL-12 2000 cells s.c. developed tumors. Experimental metastasis of IL-12 clones in nude mice was not enhanced in comparison to euthymic mice.

An increase in the number of lung nodules was observed in NK-depleted euthymic and athymic mice receiving IL-12 2000 cells i.v.; however, the highest IL-12 producer, IL-12 25'000, was completely nonmetastatic in all types of host (Fig. 3). It should be noted that IL-12 25'000 cells are capable of in vivo growth, because progressive tumors were sporadically observed in nude mice (Fig. 3). In euthymic mice subjected to repeated administrations of anti-asialo GM1, and anti-IFN-γ antibodies, we observed progressive tumor growth in two of five mice and an initial growth followed by regression in the remaining three of five mice. Tumor-free mice were protected from a subsequent challenge with parental B78H1 cells administered 2 months later.

**Immunohistochemical Analysis of Tumor Rejection.** The complete lack of tumor growth by IL-12-transduced B78H1 cells hints at a rapid elimination of tumor cells in vivo. We performed a quantitative immunohistochemical study of normal host cells infiltrating the injection site from 1–5 days after a s.c. administration of parental B78H1 cells or IL-12 clones.

A dramatic increase in specific infiltrating leukocyte populations was induced by IL-12-secreting cells, whereas other populations were attracted both by parental cells and by IL-12-engineered cells (Fig. 4). In particular, we observed an increase in the number of CD8 and NK cells, proportional to the amount of IL-12 secreted by tumor cells. Dendritic cells and eosinophils showed a threshold effect, being exclusively elicited by the high producer IL-12 25'000, whereas the low producer, IL-12 2000, was similar to parental B78H1.

The antitumoral activity of IL-12 can also be exerted via the inhibition of neoangiogenesis (20); therefore, we examined the tumor cell injection sites, particularly the tumor growth front, for the presence of microvessels, as revealed by CD34 and CD31 staining. We found that microvessels were very scarce at the site of IL-12 25'000 growth, both 3 and 5 days after cell injection, whereas parental B78H1
and CD8-depleted mice, after a short-term in vitro expansion with rIL-2, lysed the same targets used above (Fig. 7C), suggesting that NK cells were the functional effectors.

**DISCUSSION**

MHC class I loss or down-regulation is a widespread phenomenon in tumor biology. Several human tumor types exhibit a complete loss of expression of HLA antigens in a proportion of cases variable from 10 to 50%. An even higher proportion of HLA-deficient cases was reported for some tumor histotypes, for example, up to 88% in tumors of epithelial origin (1). Furthermore, the frequency of abnormalities in class I expression seems to have been underestimated in the past, because most of the published studies used mAbs that do not recognize selective losses of HLA class I alleles (2).

Defects in class I molecules may influence natural history of the tumor and may strongly counteract the potential benefit of immuno-therapy based on T cells. Immunotherapy based on non-MHC-restricted mechanisms could result more efficient on MHC-negative tumors. We chose IL-12 because it is able to activate both MHC-restricted and non-MHC-restricted antitumor responses (3). IL-12 is a potent antitumor agent; however, adverse effects were reported when the cytokine was administered systemically in mice, primates, and humans (21–24). A number of gene transfer therapeutic model systems have been developed to achieve activation of a systemic immune response by local delivery of IL-12, avoiding the toxic effects related to the systemic administration of the cytokine (8–10, 25–28).

The results reported here demonstrate that IL-12 gene transduction abolishes the tumorigenic and metastatic capacity of a MHC-negative
Therapeutic administration of IL-12-transduced cells to mice bearing nontransduced MHC-negative lung metastases resulted in a significant reduction of the metastatic load.

Multiple inflammatory and immune cells were preferentially attracted by MHC-negative tumor cells releasing IL-12. A selective increase in eosinophils, NK cells, dendritic cells, and CD8 lymphocytes was observed in the infiltrate. In turn, secondary pleiotropic cytokines were produced by infiltrating cells activated by IL-12, in particular, IFN-γ and TNF-α, which are also known inhibitors of B78H1 cell proliferation (29), and IP10, which is one of the effectors of the antiangiogenic activity of IL-12 (30).

Tumorigenic and metastatic ability of IL-12 clones was increased in nude and/or NK-depleted mice. In association with immunohistochemical and in situ results in immunocompetent mice, these data indicate that multiple mechanisms contributed to the inhibition of IL-12-secreting cells implanted in vivo. T cells were involved in the control of IL-12-secreting tumors, as evidenced by the growth of some IL-12 clones in nude mice, and NK cells played a role in the control of lung metastases. However, it must be noted that the growth of IL-12 clones was greatly inferior to that of parental cells in nude, NK-depleted, and NK-depleted nude mice. Therefore, IL-12 acted mostly through mechanisms independent of T and NK effectors. We found that in the first few days of in vivo growth, IL-12-producing cells displayed a lower angiogenic potential than parental tumors, and a strong local increase of cytokines involved in the antiangiogenic effects of IL-12, such as IFN-γ and IP10, was observed. In summary, inhibition of neoangiogenesis could be involved in the lack of in vivo growth of MHC-negative IL-12 clones.

The antitumor mechanisms activated by our MHC-negative IL-12 gene-transduced cells seem similar to those reported in other MHC-positive tumor systems (3–11, 20, 21). They include T cells, NK cells, IFN-γ, TNF, and IP10. T cells involved in our MHC-negative model were probably endowed with non-MHC-restricted activity or with immunoregulatory activity on NK function, as evidenced by in vitro cytotoxic studies.

In conclusion, tumor therapy based on gene transduction of IL-12 was effective on a MHC-negative metastatic tumor, indicating that this approach should be further pursued to devise more effective and less toxic therapies that are also applicable to MHC-defective human neoplasms.

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