Carcinogenesis vol.27 no.12 pp.2434–2441, 2006
doi:10.1093/carcin/bgl069
Advance Access publication July 19, 2006

Prophylaxis against carcinogenesis in three kinds of unestablished tumor models via IL12-gene-engineered MSCs

Xian-cheng Chen[1,2,†], Rui Wang[1,†], Xia Zhao[2], Yu-quan Wei[1,*], Min Hu[1], Yang-sheng Wang[1], Xiao-wei Zhang[1], Ru Zhang[1], Lin Zhang[1], Bin Yao[1], Lian Wang[1], Yong-qian Jia[1], Ting-ting Zeng[1], Jin-liang Yang[1], Ling Tian[1], Bing Kan[1], Xiao-juan Lin[2], Song Lei[1], Hong-xin Deng[1], Yan-jun Wen[1], Yong-qiu Mao[1] and Jiong Li[1]

[1]National Key Laboratory of Biotherapy and Cancer Center, West China Hospital and [2]Department of Gynecology and Obstetrics, Second West China Hospital, West China Medical School, Sichuan University, Guo Xue Xian No. 37, Chengdu, Sichuan 610041, People's Republic of China

*To whom correspondence should be addressed. Fax: +86 28 85250731; Email: yuquawei@vip.sina.com or xia-zhao@126.com

Mesenchymal stem cells (MSCs) were adenovirally engineered to secrete interleukin-12 (AdIL-12-MSCs) and evaluated for their anticarcinogenesis efficacy against three kinds of unestablished tumor models including B16 melanoma, LLC Lewis lung cancer and HCC hepatoma. Injection of AdIL-12-MSCs into protected mice before tumor inoculation prevented all of 12 mice in B16 preventive groups, 10 out of 12 in LLC lung cancer model and 11 out of 12 mice in HCC hepatoma model from developing tumors, whereas the control groups pre-receiving PBS were validated for 100% carcinogenesis; the tumor formation rates in free-AdIL-12 and vacant MSC groups were unveiled between ~83 and 100% even with plentiful angiogenesis and newborn lymphatic vessels, as well as distant metastases. As a novel approach, AdIL-12-MSC has revealed expected preventive effects on carcinogenesis ($P < 0.01$) with low-toxic, broad-spectrum and long-range superiorities. In conclusion, our data indicate that AdIL-12-MSC possess the potential for tropism to preclinical tumor lesions and deprives surviving or hibernating tumor cells, which have escaped from conventional treatments, of revival and recurrence.

Introduction

It is well-known that morbidities of malignant tumors have remained to increase gradually in both developing and developed countries, although human history has come into the twenty-first century and the excellent health care facilities have been progressing with contemporary culture advance. Furthermore, despite the evolutions in clinical medicine, such as improvements in early diagnosis, various new techniques including surgical, radiotherapeutic, neoadjuvant chemotherapeutic or other local and systemic therapies, mortality due to malignancy in patients is on the highest rank for various diseases (1–5). Therefore, the preclinical prevention against carcinogenesis appears to be especially practical compared with therapeutic coadministrations for an established tumor. It may be an optimum choice for underground procedure of carcinogenesis to be blockaded during incunabula phase, for hibernating tumor cells surviving various conventional administrations to be deprived from revival, or for precancerous foci to be deterred from malignant progression and reversed to normal state.

Several major human diseases, including morbidity and mortality from nutritional and infectious diseases and traffic trauma, have been controlled by the systematic prevention strategies. Among chronic diseases, the incidence of cardiovascular disease has decreased markedly as tobacco wastage has declined, cholesterol and blood pressure have been lowered, as well as, physical exercises encouraged. However, the role of prevention strategies in the overall management of cancer has been disregarded by clinical oncologists, although health care planners and society as a whole are intensely interested in this topic.

In the past decades, some chemoprevention schemes have been designed and pre-applied to tumor prevention. For example, randomized trials have shown that isotretinoin could cause regression of oral leukoplasia, and so have other researches including, for example, topical administration of β-trans-retinoic acid in cervix cancer prevention and difluoromethylornithine in colon cancer prevention (6–8). Especially, chronic intraperitoneal administration of interleukin (IL)-12 could delay mammary tumor latency but final tumor incidence was not affected, however, IL-12 systemic treatments combined with allogeneic tumor cellular vaccines could prevent the onset in progression to mammary carcinoma and lymphoproliferative malignancy (9–12). It is a pity that the defects of chemoprevention are arresting, such as obvious systemic adverse effects, continuous administration for unsustained efficacy (13,14), although some agents for cancer chemoprevention have been filtered and developed. Therefore, to overmaster morbidities of human malignancies, it has become more and more essential to exploit a novel strategy which possesses long-term efficacy with a tropism towards unestablished preneoplastic foci and with no noticeable side effect. Anticarcinogenic bioprevention, a unique gene-protective prevention mediated by cytokine-armed cellular vehicles for broad-spectrum preclinical tumors, may become an unfamiliar tumor preventive notion. Some studies have revealed that exogenously administered mesenchymal stem cells (MSCs) could target microscopic tumors during early stages of tumor development (15,16), which sounds like something of a novel tracer and provides a hint whether MSCs could be exploited as a feasible tropistic carriers of aim genes.

Abbreviations: AdIL-12-MSC, adenovirus encoding IL-12; bFGF, basic fibroblast growth factor; GFP, green fluorescent protein; HCC, hepatocellular carcinoma; IL-12, interleukin-12; LLC, Lewis lung carcinoma; MSCs, mesenchymal stem cells; NK, natural killer; PFU, plaque forming units.

[†]Both these authors contributed equally to the work.

In our study, MSCs, the adult multipotent progenitors with unique biological features over various cell lineages such as poorer immunogenicity, prosperous self-renewal and tropism into lesion stroma, were utilized as cellular vehicle of gene bioprevention. Meanwhile, IL-12, with extensive capacity to activate cytotoxic lymphocytes, inducing Th1-mediated $CD4^+$ cell differentiation, stimulate the activation of natural killer (NK) cells and the production of INF-γ (17,18), was employed as aim gene for the bioprevention. MSCs transduced by adenovirus encoding IL-12 (AdIL-12-MSC) were applied to several preclinical tumor models to investigate the protective anticarcinogenesis efficacy, as well as, the feasibility for bioprevention of carcinogenesis using genetically-engineered MSCs in mastery of human tumor.

Materials and methods

Cell lines and culture

Lewis Lung carcinoma (LLC) and B16 melanoma cells (purchased from American Type Culture Collection, ATCC, Rockville, MD) were propagated by *in vitro* passage in DMEM (Gibco BRL, Grand Island, N.Y.) supplemented with 10% of heat-inactivated fetal bovine serum (FBS; Gibco, Auckland, N.Z.), 2 mM L-glutamine and 0.1 mg/ml of Amikacin. The culture was maintained in 95% air-humidified atmosphere with 5% CO_2 at 37°C. Transplantable hepatocellular carcinoma (HCC), a cell line with poor immunogenecity, puissant invasive, metastatic and ascitogenous potential (19), was expanded by inoculating intraperitoneally 1×10^6 of the *in vivo*-passaged cells into recipient animals. The cell line was purchased from ATCC.

Mice

Female syngeneic C57BL/6 and BALB/c inbreeding mice 8–10 weeks of age were used as recipients for anticarcinogenesis model. The research protocol was reviewed and approved by the institute's Animal Care and Use Committee. During the experimental period all animals were maintained in a dedicated aseptic environment as approved by institutional protocol and guidelines.

Adenoviral vector

Adenoplasmid containing IL-12 cDNA genes (InvivoGen, San Diego, CA) under the control of cytomegalovirus promoter was constructed by using the bacterial plasmid homologous recombination pAdEsay system (20). The circular adenoviral vector DNA was set up by cloning murine IL-12 cDNA into pAdTrack-CMV Shuttle vector with GFP (9220 bp). First, the subsequent plasmid was linearized using PmeI and then cotransformed into *Escherichia coli* with adenoviral backbone plasmid pAdEasy-2 (30 767 bp), and then picked for kanamycin resistance from single plaque, digested with PacI and ethanol-precipitated. Next, linearized adenoviral plasmid was transferred with lipofectamine and plus reagent (Life Technologies) into 293 packaging cells to generate integrated adenovirus vector. Recombinant adenoviruses were purified by double CsCl (cesium chloride/ethidium brimide) gradient ultracentrifugation and sequentially dialyzed extensively against 10 mM tris (hydroxymethyl) aminomethane/1 mM $MgCl_2$ after propagated in great force in 293-H cells. Infectious unit titer was evaluated by plaque forming units (PFU).

Engineering MSCs preparation

MSCs were isolated from the mononuclear cells of femur bone marrow of female BALB/c mouse in pathogen-free condition and were separated via centrifugation over Ficoll-Hypaque separation medium at 1.088 g/cm^3 density gradient to eliminate needless cells. The monocular cells were suspended at an original planting density of 1×10^6 cells/cm^2 and kept in low-glucose DMEM with 10 U/ml bFGF in an incubator at 37°C with 5% CO_2 and 95% humidified atmosphere. After 2 days, the non-adhesive cells were weeded out after washing with PBS and the plate-adhering MSCs cohort was preserved for further expansion. Medium changes would be performed twice per week for a 14-day period of culture before first passage (21).

When the confluence of proliferating MSCs reached or surpassed ~90% between fifth and eighth passages, the cells were transduced with recombinant adenovirus vectors at a multiplicity of infection (viruses/cells) of 1500 for ~48 h according to GFP expression using Nicon TE2000-U inverted fluorescent microscope. As for single vacant MSC group with vacant vector, MSCs were incubated with adenovirus expressing a reporter gene *Lac*Z under the control of cytomegalovirus promoter.

Prophylactic experiments

Since prophylactic model was used to investigate preventive protection against carcinogenesis, pretreatment was performed prior to different tumor cell inoculations, and afterwards the tumor formation and growth were analyzed. C57BL/6 mice were used as the recipients of LLC and B16 melanoma while BALB/c inbreeding mice as the model hosts of HCC hepatoma. Mice were separated into PBS, free-AdIL-12, AdIL-12-MSC and single vacant MSC groups at random and each group consisted of 12 animals or more. Each recipient in free-AdIL-12 group was pre-infused with 5×10^8 PFU of bare virus; AdIL-12-MSC and vacant MSC groups received 5×10^5 corresponding MSCs via intraperitoneal cavity 1 week before subcutaneous implantations, in the rear left footpads (22,23), of aliquots of 3×10^5 B16 or LLC tumor cells or 3×10^6 HCC hepatoma live cells suspended in 50 µl PBS. PBS control mice did not receive special pretreatment except for the same volume of PBS. After tumor cell challenges, all mice would be under consecutive observation to see if there was any experiment-associated tumor, as well as modulation in behavior or physical appearance. Mouse bearing a tumor larger than 5 mm at the largest diameter was considered positive. Besides gross observations, histological verdict for tumorigenesis was verified by two pathologists in a blinded manner. Tumor growth was monitored once per week by measuring the two perpendicular diameters using a caliper. Animals without tumorigenesis received a second challenge through an inoculation of a double dose of the parent tumor cells on their backsides, 2–3 months later, in order to validate the anticarcinogenesis results.

Section preparation and immunohistochemistry staining

Specimens of primary tumor lesions with adjacent tissues, relevant internal organs and tumor-draining lymph nodes were harvested and fixed in 4% of neutral formalin for paraffin sectioning of H&E. For immunohistochemistry analysis of lymph-angiogenesis with carcinogenesis, sections were probed with polyclonal goat antimouse LYVE-1 (1:200; Santa Cruz Biotechnology, CA), with which the sections would be incubated overnight at 4°C, followed by incubation with biotinylated rabbit anti-goat IgG secondary antibodies at 37°C for 45 min, washed with slight agitation in PBS for 10 min, and incubated with avidin–biotin–horseradish peroxidase complex at 37°C for 45 min. Cell nuclei were gently counterstained with reformative Gill's hematoxylin and slides were dehydrated and mounted.

Determination of long-lasting serum levels of IL-12

After MSCs/AdIL-12 pre-infusion, serum samples were collected from the lateral tail veins or retro-orbital sinus at Week 1 and again at Week 5. Serum IL-12 levels were detected in triplicate by using sandwich ELISA (enzyme-linked immunosorbent assay kit; R&D Systems). Briefly, a 96-well microtiter plate was coated with monoclonal antibodies against IL-12 as the capture antibody (5 µg/ml) overnight at 4°C. Plate contents were emptied and then washed twice with PBS/Tween-20 solution, and non-specific binding was blocked using 5% BSA in PBS for 120 min at room temperature. The blocking buffer was removed and wells were washed four times. Serial dilutions of serum samples were added to the coated plate the next day and incubated at ambient temperature for 60 min. A biotinlated rat antimouse IL-12 polyclonal antibody was used as a detection antibody as per the protocols of kit manufacturer. The plates were washed eight times and 100 µl of 2,2′-azinobis(3-ethylbenzo-thiazoline-6-sulfonic acid) was added per well. Plates were read at an optical density of 405 nm. A standard IL-12 curve containing known concentration of the cytokine was performed.

Toxicity evaluation

To investigate potential side effects or toxicity in the pretreated mice, they were observed continuously for relevant indexes such as weight loss, ruffled fur, diarrhea, anorexia, cachexia, skin ulceration or toxic deaths. The tissues of heart, liver, spleen, lung, kidney, brain, and so on were fixed in 4% neutral buffered formalin solution and embedded in paraffin. Slices of 3–5 µm were stained with hematoxylin and eosin (HE) and observed by two pathologists in a blinded manner.

Statistical analysis

Experimental data would be analyzed by ANOVA based on arcsine-transformed proportion. The statistical differences in oncogenesis rates would be confirmed using log rank test and Kaplan–Meier method (24). P values <0.05 were considered significant.

Results

In vitro expression of MSC-AdIL-12

MSCs expressed GFP with ~75 and ~100% of luminescence rates at 36 and 48 h, respectively, after transduction with recombinant AdIL-12 at a multiplicity of infection (viruses/cells) of 1500 (Figure 1A–D). Subsequently, although almost all cells emitted green fluorescence under cultivated plate-adhering condition, the MSCs were pelleted in a middle speed centrifuge and then stored in −80°C by suspending in 90% FBS with 10% DMSO. Over half a year or more, the AdIL-12-transduced MSCs were resuscitated at 40°C water-bathing and recultivated in low-glucose DMEM. We found that not only were the rates of fluorescent cells not less than that of the cells before they were frozen but the luminescence intensity appeared to be not weaker than before frozen. The percentage of fluorescence-bearing genetically manipulated MSCs remained ~100% after thawing from the hibernation state (Figure 1E and F). It seemed possible that genetically-engineered MSCs could be exploited into instant models, kept in lower temperature, and unfrozen as quickly as possible when required clinically, just like cryo-fresh vaccines.

Impact of AdIL-12-MSC on oncogenesis

Control groups pre-receiving PBS were validated for 100% carcinogenesis in all three kinds of tumor models; carcinogenesis rates in free-AdIL-12 and vacant MSC groups were unveiled between ~83 and 100%; whereas, injection of AdIL-12-MSCs into protected mice prior to tumor inoculation resulted in complete determents of carcinogenesis in all the mice of the B16 preventive group, 11 out of the 12 mice in the HCC hepatoma model and 10 out of 12 in the LLC model ($P < 0.01$), and those mice with no tumorigenesis all stood against the re-challenge of double doses of parent tumor cells on their backsides without any tumor formation (Figure 2).

Prevention of B16 oncogenesis

For the B16 melanoma preventive model, none of the 12 mice in AdIL-12-MSC group developed tumors within 2 months. In contrast, all of the mice receiving free-AdIL-12 or PBS and 10 out of 12 mice receiving vacant MSC had severally sent forth noticeable tumors ~5 days post-inoculation with a survival within 30 days for PBS group, 35 days for free-AdIL-12 group and 40 days for vacant MSC group (Figure 3A). Similar preventive results were obtained in two independent experiments. In free-AdIL-12-pretreated group, numerous B16 melanoma cells proliferated around newborn LYVE-1$^+$ lymphatic vessels and formed rosette-like models (Figure 3B) with metastasis formation in lung (Figure 3C) just 4 weeks after tumor inoculation in the footpads.

Prevention of HCC hepatoma carcinogenesis

For the HCC hepatoma preventive model, 11 out of the 12 mice in AdIL-12-MSC group did not develop tumors during the 3 months. In contrast, all of the mice in PBS and vacant MSC groups and 10 out of 12 mice receiving free-AdIL-12 had presented noticeable tumors ~7 days post-inoculation with a longest life span within 65 days for vacant MSC and free-AdIL-12 groups and 75 days for PBS group (Figure 4A). Similar protective results were achieved in two independent experiments. In free-AdIL-12 group, there were plentiful LYVE-1$^+$ newborn lymphatic vessels with some dilated lumens among the tumor cells, especially in the fencing zone (Figure 4B) and obvious metastatic deposits in liver at the end of experiment (Figure 4C).

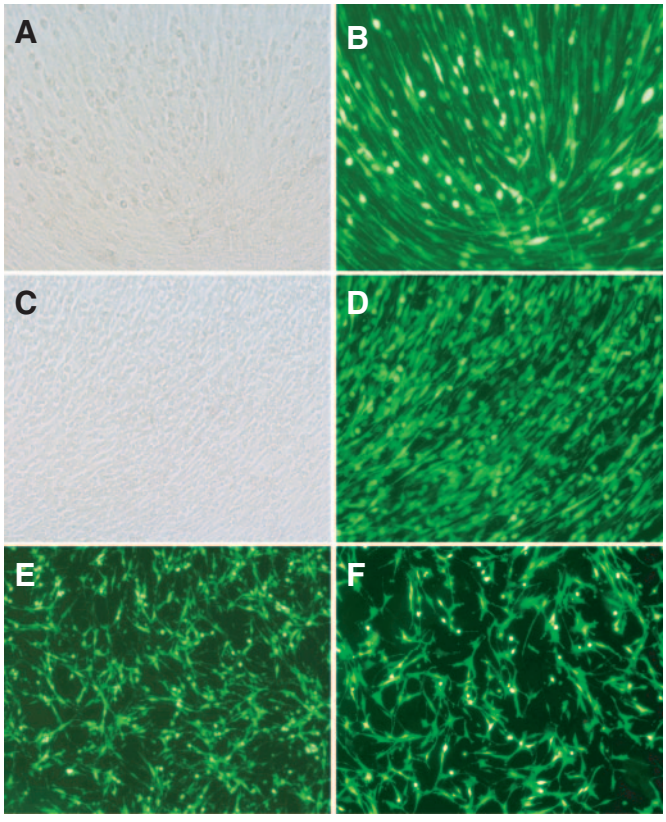

Fig. 1. The phase contrast and fluorescence images of transduced MSCs. MSCs expressed GFP with ~ 75 and ~100% of luminescence rates at 36 and 48 h, respectively after transduction with recombinant AdIL-12 at a multiplicity of infection (viruses/cells) of 1500. (**A**) and (**B**) demonstrate the same visual fields, as do (**C**) and (**D**). The ready-to-use cells revealed re-anchorage-dependent growth and expressed green fluorescence from almost all of the cells 24 h after being unfrozen quickly from −80°C refrigerator and recultivated in low-glucose DMEM, just before inoculating the animals (**E** and **F**).

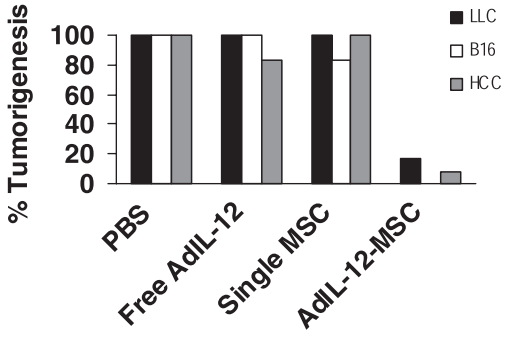

Fig. 2. Impact of AdIL-12-MSC on oncogenesis. Control groups pre-receiving PBS were validated for 100% carcinogenesis in all the three kinds of tumor models; the oncogenesis rates in free-AdIL-12 and single vacant MSC groups were unveiled between ~83 and 100%; whereas, infusions of AdIL-12-MSCs prior to tumor inoculation did prevent completely oncogenesis in all the mice in the B16 preventive groups, in 11 out of 12 mice in the HCC hepatoma model and 10 out of 12 in the LLC lung cancer model ($P < 0.01$). Animals without tumorigenesis received a second challenge through an inoculation of a double dose of the parent tumor cells on their backsides, 2–3 months later.

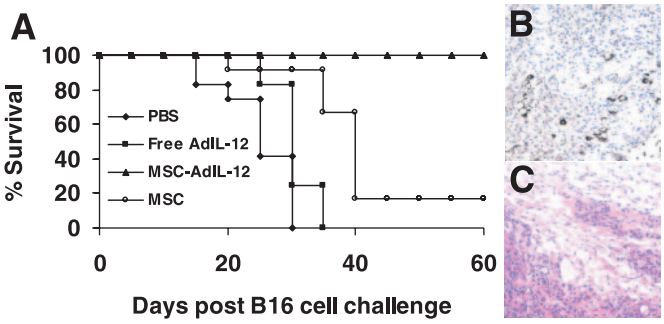

Fig. 3. Prevention of oncogenesis in B16 melanoma model. None of the 12 mice in AdIL-12-MSC group developed tumors. In contrast, all of the mice receiving free-AdIL-12 or PBS, and 10 out of 12 mice receiving single vacant MSCs severally sent forth noticeable carcinogenesis ~5 days post-inoculation and only survived 30–40 days (**A**). In free-AdIL-12-pretreated group, numerous B16 melanoma cells proliferated around LYVE-1^+ newborn lymphatic vessels and formed some sunflower models (**B**) with metastasis in lung (**C**) 4 weeks after tumor inoculation in the footpads.

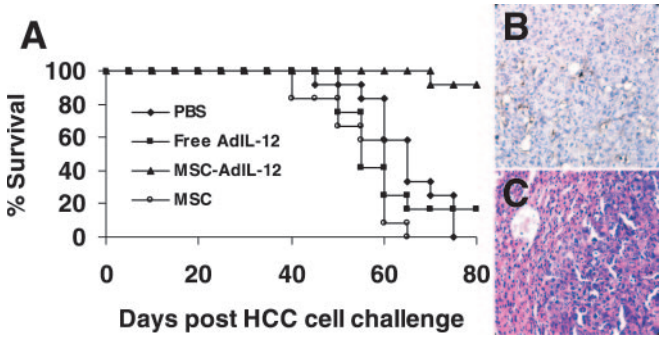

Fig. 4. Prevention of carcinogenesis in HCC hepatoma model. Out of the 12 mice in AdIL-12-MSC group 11 did not develop tumors. In contrast, all of the mice in the PBS and vacant MSC groups, and 10 out of 12 mice receiving free-AdIL-12 had presented noticeable tumors ~7 days post-inoculation with the longest life span of 65–75 days (**A**). In free-AdIL-12 group, there were plentiful newborn lymphatic vessels with some dilated lumens among the tumor cells, especially at the fencing zone (**B**) and metastatic deposits in liver 8 weeks after tumor inoculation in the footpads (**C**).

Prevention of LLC lung cancer carcinogenesis

As for the LLC lung cancer preventive model, only 2 out of the 12 mice receiving AdIL-12-MSC developed tumors during the 3 months. However, noticeable carcinogenesis had respectively emerged in all of the mice of PBS, vacant MSC and free-AdIL-12 groups 1 ~ 2 weeks post-inoculation and their longest life span did not exceed 45 days for PBS group and 55 days for vacant MSC and free-AdIL-12 groups (Figure 5A). Similar protective results were obtained in two independent experiments. In vacant MSC groups, numerous lung cancer cells proliferated around LYVE-1^+ newborn lymphatic vessels (Figure 5B) and around newborn blood vessels (Figure 5C) 4 weeks after tumor inoculation in the footpads.

Lymph-angiogenesis in control groups

There were plentiful newborn lymphatic vessels among the tumor cells in PBS, free-AdIL-12 (Figures 3B and 4B) and vacant MSC groups (Figure 5B), indicating the overgrowth of LYVE-1^+ lymphatic vessels with carcinogenesis. There were also abundant newborn microvessels around the tumor cells in PBS, free-AdIL-12 and vacant MSC groups (Figure 5C).

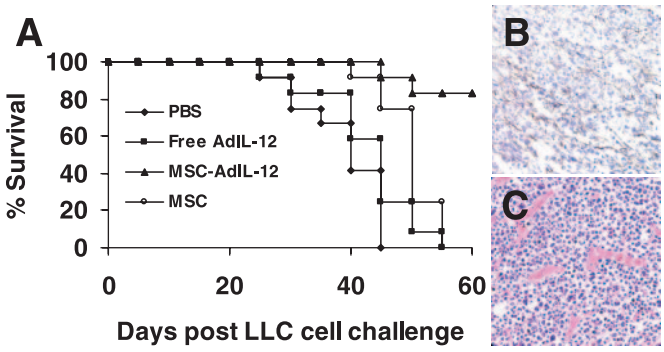

Fig. 5. Prevention of carcinogenesis in LLC lung cancer model. Only 2 out of the 12 mice receiving AdIL-12-MSC developed tumors. However, all of the mice in the other three groups had noticeable carcinogenesis 1 ~ 2 weeks post-inoculation and their longest life span did not exceed 55 days (**A**). In vacant MSC groups, analyses by immunohistochemistry demonstrated that numerous cancer cells proliferated around newborn lymphatic vessels (**B**) and blood vessels (**C**) 4 weeks after tumor inoculation in the footpads.

Prolonged IL-12 expression by AdIL-12-MSCs

The long-term gene expression levels of IL-12 in different models were examined by measuring the serum concentrations of the cytokine at Weeks 1 and 5, after AdIL-12-MSCs infusion, using sandwich ELISA (Figure 6). IL-12 levels decreased from ~60 pg/ml to ~≤10 pg/ml from Week 1 through Week 5 after infusion of free-AdIL-12 ($P < 0.05$); while IL-12 levels were maintained between 20 and 25 pg/ml from Week 1 to 5 after pretreatment with AdIL-12-MSC, where there was no significant difference between Weeks 1 and 5 after pretreatment ($P > 0.05$). PBS and vacant MSC administration did not result in any elevation of serum IL-12 (<10 pg/ml).

Toxicity observation

To evaluate the health status of mice pretreated with AdIL-12-MSCs infusion, weight of mice was monitored once every 10 days throughout the whole experiment and considered a parameter for evaluation of systemic well-being, anorexia, or cachexia. It was plotted at regular intervals and no significant differences in weights were found among the four groups (Figure 7). The weight curve of AdIL-12-MSCs group paralleled very closely that of the control PBS and single vacant MSC groups. All animals thrived after pretreatments except for a 1 to 2 week long episode of some weight loss accompanied by high IL-12 serum levels ~60 pg/ml (Figure 6) in control animals soon after the pretreatment of free-AdIL-12. No adverse effects in other gross measures such as ruffled fur, skin tenting or ulcerations, or toxic death were observed in the AdIL-12-MSCs group. Furthermore, toxic pathologic changes in liver, lungs, kidneys, spleen, brain, heart, pancreas, intestine, or bone marrow were not detected by microscopic examination.

Comparative analysis of tumor growth

Although free-AdIL-12 could retard tumor growth to some degree in B16 melanoma and HCC hepatoma cohorts (Figure 8A and B), there were no significant differences among them ($P > 0.05$). free-AdIL-12 could hardly impose visible inhibitory effect on tumor growth of LLC lung cancer cohort (Figure 8C).

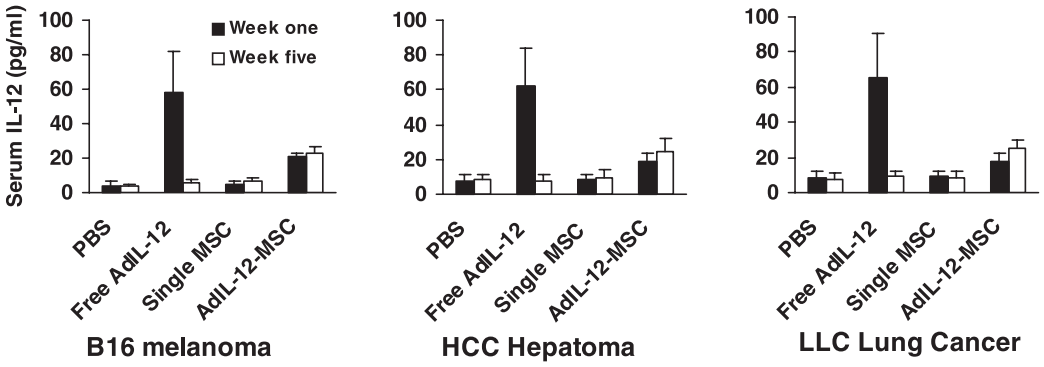

Fig. 6. Serum IL-12 levels in different time points were detected via sandwich ELISA system. IL-12 levels decreased from ~60 pg/ml in Week 1 to ~≤10 pg/ml in Week 5 after injection of free-AdIL-12; meanwhile serum IL-12 levels were maintained between 20 and 25 pg/ml after pretreatment with AdIL-12-MSC. PBS and single vacant MSC administration did not enhance serum IL-12 level (<10 pg/ml). Data were presented as means ± SD. Each group contained six mice.

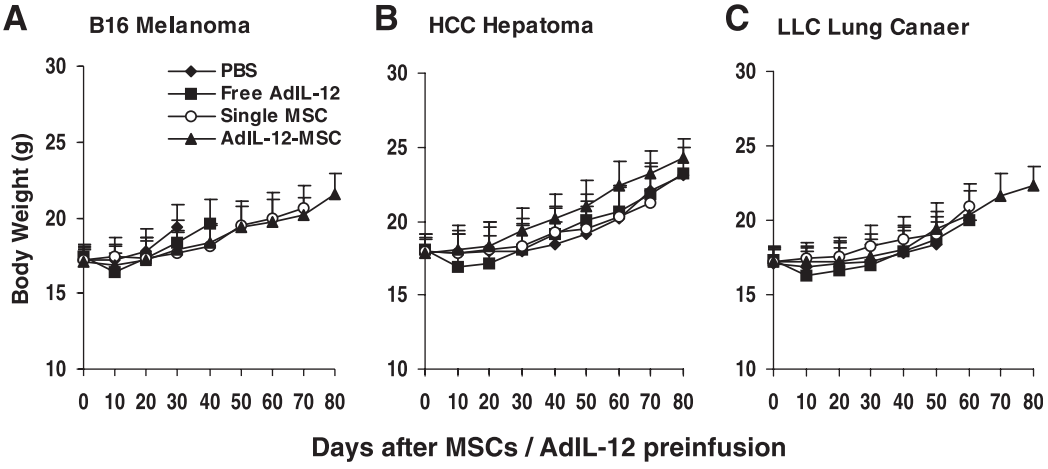

Fig. 7. Lack of toxicity-dependent weight loss in mice pretreated with AdIL-12-MSCs. Body weights were plotted at regular intervals and the curve of AdIL-12-MSCs group paralleled very closely that of the control group with no significant differences among the four groups. All animals thrived after pretreatments with the exception of a 1–2-week-long episode of some weight gain retardation accompanied by serum levels of high IL-12, ~60 pg/ml, (as shown in Figure 6) in control animals pretreated with free-AdIL-12.

Discussion

Since the morbidities and mortalities of malignancies remain to be controlled in the forthcoming years people at risk of malignancies and of recurrence deserve to benefit from appropriate, effective and safe preventive approaches, if available. Besides avoiding the evitable causes for normal or asymptomatic individuals, screening and early detection in high-risk populations, as well as, chemoprevention for individuals with evidence of preneoplastic progression, but without frank malignancy, we still need to obtain other ways for the management of preneoplasia to blockade the pathologic process early on. To exploit an ideal broad-spectrum and long-term bioprevention platform, with a tropism towards unestablished preneoplastic foci to overmaster oncogenesis and at the same time with no obvious or unacceptable side-effects, has become more and more indispensable (25–29). So in our present study, certain observations on three kinds of tumor preventive models have been made in relevant experiments concerning the genetically modified MSCs as vehicles for anticarcinogenesis gene therapy, which demonstrated the evidences that MSCs packed with AdIL-12 could validly trigger prophylactic immunity against tumorigenesis challenge in three models. In B16 melanoma model, none of the mice pre-receiving AdIL-12-MSC presented tumorigenesis. As for HCC hepatoma and LLC lung cancer models, 11 and 10 mice, respectively out of the 12 mice in MSC-AdIL-12 groups did not develop tumors. In contrast, all of the mice in control PBS and >83% mice pretreated with single vacant MSCs or free-AdIL-12 had noticeable tumorigenesis ~5–12 days post-inoculation. It seemed as if these protective tumor biopreventions were very provocative and acceptable.

The brief principle of preventive mechanisms may be explained as follows. IL-12 appears to be located in the central joint connecting non-specific immune surveillance functions with the exertion and engagement of specific T cell-mediated immune responses (30–32). Although it has been well-known that IL-12 is an immunoregulatory cytokine that plays an active role in cell-mediated immunity against established tumors (33), when IL-12/Ad-12 is directly injected into living organism, portions of it will be destroyed by the immune system; and especially in order to obtain its antitumor activity, IL-12 has to be used in high doses with repeated administrations which is usually accompanied with serious adverse effects (34). Since MSCs possess unique

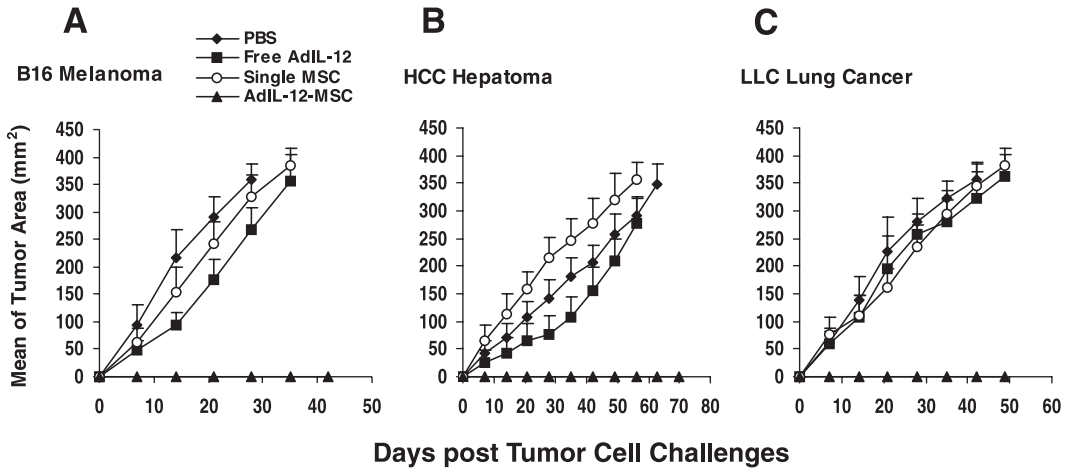

Fig. 8. Comparative analysis of tumor formation and growth in different control groups. free-AdIL-12 could retard tumor growth to some extent in B16 melanoma (**A**) and HCC hepatoma models (**B**). However, there were no significant differences ($P > 0.05$). free-AdIL-12 could hardly impose observable inhibitive effect on tumor growth of LLC lung cancer model (**C**).

biological features such as poorer immunogenicity among various types of cells, tropism towards established tumor lesions (35), as well as, prosperous self-renewal and differentiations into various cell lineages including vasculature of developing tumor (36), we used MSCs, a relatively perfect candidate of cellular vehicles so far (37,38), as cellular vehicle, which could enable IL-12 to escape from being destroyed due to entering the circulation system and being accompanied by apparent adverse effects owing to interfering spontaneously with other systems and thereby improve its pharmacokinetics wonderfully. Our present experiments further hint that AdIL-12-MSC possesses the potential for tropism towards unestablished tumor foci or developing preneoplastic lesions to prevent residue or hibernating tumor cells having survived conventional administrations from revival or recurrence. Their possible anticarcinogenic molecular mechanism is not clear but may be associated with multiple actions including (i) production of IFN-γ and IFN-inducible protein-10 at tropistic site, in draining lymph nodes and in spleen (39–48); (ii) abduction of apoptosis within unestablished tumor foci by providing a unique array of signals leading to activation of the Fas/FasL apoptosis pathway (49); (iii) arrest of multistep carcinogenesis in distinct target tissues; via different lymphocyte subsets, primarily responsible for cytotoxic activities among local tissues such as NK and $CD4^+$ T cells, to limit or prevent transition of preneoplastic lesions to overt carcinoma(50); (iv) inhibition of preneoplastic neovascularization, thus depriving preclinical malignant cells of nutrient uptake for multiplication (51,52).

Lower and steady serum levels of IL-12 with no significant difference from Week 1 through Week 5 after pretreatment and no apparent toxic effects during entire experiments may be due to the directional engagements of pre-receiving AdIL-12-MSC at arrested preneoplastic lesions instead of systemic expression, which can offer ascendency in reducing potential systemic side-effects and in the absence of toxicity (53–55). The 1–2-week-long episode of some weight loss in control animals, soon after being pretreated with free-AdIL-12, may be due to high serum levels of IL-12, ∼60 pg/ml. It was difficult to maintain the expression level of IL-12 without repeated injections of AdIL-12, like the situation in free-AdIL-12 group, since the dissociating-AdIL-12 would be easily eliminated by the immune system in living organism.

In conclusion, as a novel bioprevention approach, AdIL-12-MSC has revealed expected protective anticarcinogenesis impact on interesting preneoplastic lesions. The identification of AdIL-12-MSC as a targeted bioprevention agent may indicate a development of a favorite strategy with low-toxic and long-lasting virtue for the preventive application of broad-spectrum preclinical malignancy in humans. Maybe the best treatment of malignant disease is its prevention, and further efforts should be dedicated to the identification and application of more such biological agents or approaches.

Acknowledgements

This investigation was supported by National Basic Research Program of China (2001CB510001, 2004CB518800), the projects of National Natural Science Foundation of China, and National 863 Program.

Conflict of Interest Statement: None declared.

References

1. Cowen,R.L., Williams,K.J., Chinje,E.C., Jaffar,M., Sheppard,F.C.D., Telfer,B.A., Wind,N.S. and Stratford,I.J. (2004) Hypoxia targeted gene therapy to increase the efficacy of tirapazamine as an adjuvant to radiotherapy: reversing tumor radioresistance and effecting cure. *Cancer Res.*, **64**, 1396–1402.
2. Martenson,J.A., Willett,C.G., Sargent,D.J. *et al.* (2004) Phase III study of adjuvant chemotherapy and radiation therapy compared with chemotherapy alone in the surgical adjuvant treatment of colon cancer: results of intergroup protocol. *J. Clin. Oncol.*, **22**, 3277–3283.
3. Malaisrie,S.C., Untch,B., Aranha,G.V., Mohideen,N., Hantel,A. and Pickleman,J. (2004) Neoadjuvant chemoradiotherapy for locally advanced esophageal cancer: experience at a single institution. *Arch. Surg.*, **139**, 532–539.
4. Spiro,S.G. and Silvestri,G.A. (2005) One hundred years of lung cancer. *Am. J. Respir. Crit. Care Med.*, **172**, 523–529.
5. Mulshine,J.M. (2005) Clinical issues in the management of early lung cancer. *Clin. Cancer Res.*, **11**, 4993–4998.
6. Meyskens,F.L. Jr, Surwit,E., Moon,T.E. *et al.* (1994) Enhancement of regression of cervical intraepithelial neoplasia II (moderate.dysplasia) with topically applied all-trans-retinoic acid: a randomized trial. *J. Natl Cancer Inst.*, **86**, 539–543.

7. Lippman,S.M., Batsalis,J.G., Toth,B.B. *et al.* (1993) Comparison of low-dose isotretinoin with beta-carotene to prevent oral carcinogenesis. *N. Engl. J. Med.*, **328**, 15–20.
8. Meyskens,F.L.Jr, Gerner,E.W., Emerson,S. *et al.* (1998) Effect of difluoromethylornithine on rectal mucosal levels of polyamines in a randomized double-blind trial for colon cancer prevention. *J. Natl Cancer Inst.*, **90**, 1212–1218.
9. Nicoletti,B.K.G., Carlo,E.D., Cavallo,F., Landuzzi,L., Melani,C., Giovarelli,M., Rossi,I., Nanni,P. and Giovanni,C.D. (1998) Interleukin 12-mediated prevention of spontaneous mammary adenocarcinomas in two lines of Her-2/neu transgenic mice. *J. Exp. Med.*, **188**, 589–596.
10. Nanni,P., Nicoletti,G., Giovanni,C.D. *et al.* (2001) Combined allogeneic tumor cell vaccination and systemic interleukin 12 prevents mammary carcinogenesis in her-2/neu transgenic mice. *J. Exp. Med.*, **194**, 1195–1206.
11. Curt,A., Parenza,M. and Colombo,M.P. (2003) Autologous and MHC class I-negative allogeneic tumor cells secreting IL-12 together cure disseminated A20 lymphoma. *Blood*, **101**, 568–575.
12. Liu,J.G., Xiang,Z.Y. and Ma,X.J. (2004) (Role of IFN regulatory factor-1 and IL-12 in immunological resistance to pathogenesis of *N*-methyl-*N*-nitrosourea-induced T lymphoma. *J. Immunol.*, **173**, 1184–1193.
13. Tsao,A.S., Edward,S., Kim,E.S. and Hong,W.K. (2004) Chemoprevention of cancer. *CA Cancer J. Clin.*, **54**, 150–180.
14. Goodin,S. and Shiff,S.J. (2004) NSAIDs for the chemoprevention of oral cancer: promise or pessimism? *Clin. Cancer Res.*, **10**, 1561–1564.
15. Van Damme,A., Vanden Driessche,T. and Collen,D. (2002) Bone marrow stromal cells as targets for gene therapy. *Curr. Gene Ther.*, **2**, 195–209.
16. Hung,S.C., Deng,W.P., Yang,W.K. *et al.* (2005) Mesenchymal stem cell targeting of microscopic tumors and tumor stroma development monitored by noninvasive in vivo positron emission tomography imaging. *Clin. Cancer Res.*, **11**, 7749–7756.
17. Shrayer,D.P., Bogaars,H., Cole,B., Wolf,S.F. and Wanebo,H.J. (2002) Capacity of murine IL-12 to inhibit the development of primary melanoma tumors and to prevent lung metastases in the melanoma-challenged mice. *J. Exp. Thera. Oncol.*, **2**, 93–102.
18. Yoon,T.J., Yoo,Y.C., Kang,T.B., Song,S.K., Lee,K.B., Her,E., Song,K.S. and Kim,J.B. (2003) Antitumor activity of the Korean mistletoe lectin is attributed to activation of macrophages and NK cells. *Arch. Pharm. Res.*, **26**, 861–867.
19. Wang,Z., Qiu,S.J., Ye,S.L., Tang,Z.Y. and Xiao,X. (2001) Combined IL-12 and GM-CSF gene therapy for murine hepatocellular carcinoma. *Cancer Gene Ther.*, **8**, 751–758.
20. He,T.C., Zhou,S., Costa,L.T.D., Yu,J., Kinzler,K.W. and Vogelstein,B.A. (1998) simplified system for generating recombinant adenoviruses. *Proc. Natl Acad. Sci. USA*, **95**, 2509–2514.
21. Peister,A., Mellad,J.A., Larson,B.L., Hall,B.M., Gibson,L.F. and Prockop,D.J. (2004) Adult stem cells from bone marrow (MSCs) isolated from different strains of inbred mice vary in surface epitopes, rates of proliferation, and differentiation potential. *Blood*, **103**, 1662–1668.
22. Fischer,A.H., Taysavang,P. and Jhiang,S.M. (2003) Nuclear envelope irregularity is induced by RET/PTC during interphase. *Am. J. Pathol.*, **163**, 1091–1100.
23. Hiraoka,K., Yamamoto,S., Otsuru,S., Nakai,S., Tamai,K., Morishita,R., Ogihara,T. and Kaneda,Y. (2004) Enhanced tumor-specific long-term immunity of hemagglutinating virus of Japan-mediated dendritic cell-tumor fused cell vaccination by coadministration with CpG oligodeoxynucleotides. *J. Immunol.*, **173**, 4297–4307.
24. Ebert,O., Harbaran,S., Shinozaki,K. and Woo,S.L. (2005) Systemic therapy of experimental breast cancer metastases by mutant vesicular stomatitis virus in immune-competent mice. *Cancer Gene Thera.*, **12**, 350–358.
25. Finn,O.J. (2003) Cancer vaccines: between the idea and the reality. *Nat. Rev. Immunol.*, **3**, 630–641.
26. Lollini,P.L. and Forni,G. (2003) Cancer immunoprevention: tracking down persistent tumor antigens. *Trends Immunol.*, **24**, 62–66.
27. Finn,O.J. and Forni,G. (2002) Prophylactic cancer vaccines. *Curr. Opin. Immunol.*, **14**, 172–177.
28. Lollini,P.L. and Forni,G. (2002) Antitumor vaccines: is it possible to prevent a tumor? *Cancer Immunol. Immunother.*, **51**, 409–416.
29. Forni,G., Lollini,P.L., Musiani,P. and Colombo,M.P. (2000) Immunoprevention of cancer: is the time ripe? *Cancer Res.*, **60**, 2571–2575.
30. Kobayashi,M., Fitz,L., Ryan,M., Hewick,R.M., Clark,S.C., Chan,S., Loudon,R., Sherman,R., Perussia,B. and Trinchieri,G. (1989) Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J. Exp. Med.*, **170**, 827–845.
31. Wolf,S.F., Temple,P.A., Kobayashi,M. *et al.* (1991) Cloning of cDNA for natural killer cell stimulatory factor, a heterodimeric cytokine with multiple biologic effects on T and natural killer cells. *J. Immunol.*, **146**, 3074–3081.
32. Trinchieri,G. and Gerosa,F. (1996) Immunoregulation by interleukin-12. *J. Leukocyte Biol.*, **59**, 505–511.
33. Fan,H., Walters,C.S., Dunston,G.M. and Tackey,R. (2002) IL-12 plays a significant role in the apoptosis of human T cells in the absence of antigenic stimulation. *Cytokine*, **19**, 126–137.
34. Cohen,J. (1995) Clinical trials: IL-12 deaths: explanation and a puzzle. *Science*, **270**, 908.
35. Serakinci,N., Guldberg,P., Burns,J.S., Abdallah,B., Schrodder,H., Jensen,T. and Kassem,M. (2004) Adult human mesenchymal stem cell as a target for neoplastic transformation. *Oncogene*, **23**, 5095–5098.
36. Isner,J.M. and Asahara,T. (1999) Angiogenesis and vasculogenesis as therapeutic strategies for postnatal neovascularization. *J. Clin. Invest.*, **103**, 1231–1236.
37. Studeny,M., Marini,F.C., Champlin,R.E., Zompetta,C., Fidler,I.J. and Andreeff,M. (2002) Bone marrow-derived mesenchymal stem cells as vehicles for interferon-beta delivery into tumor. *Cancer Res.*, **62**, 3603–3608.
38. Deans,R.J. and Moseley,A.B. (2000) Mesenchymal stem cells: biology and potential clinical uses. *Exp. Hematol.*, **28**, 875–884.
39. Nastala,C.L., Edington,H.D., McKinney,T.G. *et al.* (1994) Recombinant IL-12 administration induces tumor regression in associated with IFN-production. *J. Immunol.*, **153**, 1697–1706.
40. Fernandez,N.C., Levraud,J.P., Haddada,H., Perricaudet,M. and Kourilsky,P. (1999) High frequency of specific CD8+ T cells in the tumor and blood is associated with efficient local *IL-12* gene therapy of cancer. *J. Immunol.*, **162**, 609–617.
41. Saffran,D.C., Horton,H.M., Yankauchas,M.A., Anderson,D., Barnhart,K.M., Abai,A.M., Hobart,P., Manthorpe,M., Norman,J.A. and Parker,S.E. (1998) Immunotherapy of established tumors in mice by intratumoral injection of interleukin-2 plasmid DNA: induction of CD8+ T-cell immunity. *Cancer Gene Ther.*, **5**, 321–330.
42. Rakhmilevich,A.L., Turner,J., Ford,M.J., McCabe,D., Sun,W.H., Sondel,P.M., Grota,K. and Yang,N.-S. (1996) Gene gun-mediated skin transfection with *interleukin 12* gene results in regression of established primary and metastatic murine tumors. *Proc. Natl Acad. Sci. USA*, **93**, 6291–6296.
43. Tannenbaum,C.S., Tubbs,R., Armstrong,D., Finke,J.H., Bukowski,R.M. and Hamilton,T.A. (1998) The CXC chemokines IP-10 and Mig are necessary for IL-12-mediated regression of the mouse RENCA tumor. *J. Immunol.*, **161**, 927–932.
44. Lode,H.N., Dreier,T., Xiang,R., Varki,N.M., Kang,A.S. and Reisfeld,R.A. (1998) Gene therapy with a single chain interleukin 12 fusion protein induces T cell-dependent protective immunity in a syngeneic model of murine neuroblastoma. *Proc. Natl Acad. Sci. USA*, **95**, 2475–2480.
45. Tannenbaum,C.S., Wicker,N., Armstrong,D., Tubbs,R., Finke,J., Bukowski,R.M. and Hamilton,T.A. (1996) Cytokine and chemokine expression in tumors of mice receiving systemic therapy with IL-12. *J. Immunol.*, **156**, 693–699.
46. Alatrash,G., Hutson,T.E., Molto,L. *et al.* (2004) Clinical and immunologic effects of subcutaneously administered interleukin-12 and interferon alfa-2b: phase I trial of patients with metastatic renal cell carcinoma or malignant melanoma. *J. Clin. Oncol.*, **22**, 2891–2900.
47. Gately,M.K., Warrier,R.R. and Honasoge,S. (1994) Administration of recombinant IL-12 to normal mice enhances cytolytic lymphocyte activity and induces production of IFN- *in vivo*. *Int. Immunol.*, **6**, 157–167.
48. Manetti,R., Gerosa,F. and Giudizi,M.G. (1994) Interleukin 12 induces stable priming for interferon (IFN-) producing during differentiation of human T helper (Th) cells and transient IFN- production in established Th2 cells clones. *J. Exp. Med.*, **179**, 1273–1283.
49. Wigginton,J.M., Park,J.W., Gruys,M.E. *et al.* (2001) Complete regression of established spontaneous mammary carcinoma and the therapeutic prevention of genetically programmed neoplastic transition by IL-12/pulse IL-2: induction of local T cell infiltration, fas/fas ligand gene expression, and mammary epithelial apoptosis. *J. Immunol.*, **166**, 1156–1168.
50. Nakagawa,R., Nagafune,I., Tazunoki,Y. *et al.* (2001) Mechanisms of the antimetastatic effect in the liver and of the hepatocyte injury induced by α-galactosylceramide in mice. *J. Immunol.*, **166**, 6578–6584.
51. Yao,L., Sgadari,C., Furuke,K., Bloom,E.T., Teruya-Feldstein,J. and Tosato,G. (1999) Contribution of natural killer cells to inhibition of angiogenesis by interleukin-12. *Blood*, **93**, 1612–1621.

52. Voest,E.E., Kenyon,B.M., O'Reilly,M.S., Truitt,G., D'Amato,R.J. and Folkman,J. (1995) Inhibition of angiogenesis *in vivo* by interleukin 12. *J. Natl Cancer Inst.*, **87**, 581–586.
53. Hess,S.D., Egilmez,N.K., Bailey,N. *et al.* (2003) Human CD4+ T cells present within the microenvironment of human lung tumors are mobilized by the local and sustained release of IL-12 to kill tumors *in situ* by indirect effects of IFN-γ. *J. Immunol.*, **170**, 400–412.
54. Nakayama,Y., Inoue,Y., Nagashima,N. *et al.* (2004) Relationships between local and systemic expression of interleukin-12 and plasma levels of vascular endothelial growth factor in patients with gastric cancer. *Anticancer Res.*, **24**, 3289–3294.
55. Broderick,L., Yokota,S.J., Reineke,J. and Mathiowitz,E. (2005) Human CD4+ effector memory T cells persisting in the microenvironment of lung cancer xenografts are activated by local delivery of IL-12 to proliferate, produce IFN-, and eradicate tumor cells. *J. Immunol.*, **174**, 898–906.

Received October 4, 2005; revised March 4, 2006; accepted April 21, 2006