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Optimization of the Tet-on System To Regulate Interleukin 12 Expression in the Liver for the Treatment of Hepatic Tumors

Maider Zabala,[1] Lin Wang,[2] Ruben Hernandez-Alcoceba,[1] Wolfgang Hillen,[3] Cheng Qian,[1] Jesus Prieto,[1] and M. Gabriela Kramer[1]

[1]Division of Hepatology and Gene Therapy, School of Medicine, Fundacion para la Investigacion Medica Aplicada (FIMA), University of Navarra, Pamplona, Spain; [2]Jilin University, Department of Pathology, Changchun, China; and [3]Institüt für Mikrobiologie, Biochemie und Genetik, Friedrich-Alexander Universität, Erlangen-Nurnberg, Germany

ABSTRACT

Interleukin 12 (IL-12) is a potent antitumoral cytokine, but it can be toxic at high doses. Therapy of liver tumors might benefit from the use of vectors enabling tight control of IL-12 expression in hepatic tissue for long periods of time. To this aim, we have improved the Tet-on system by modifying the minimal region of the inducible promoter and adjusting the level of the *trans*-activator using liver-specific promoters with graded activities. The resulting vectors allowed hepato-specific gene regulation with lower basal activity and higher inducibility compared with the original system in the absence of repressor molecules. The basal and final protein levels depend on the strength of the promoter that directs the transcripcional activator as well as the relative orientation of the two genes in the same plasmid. We have selected the construct combining minimal leakage with higher level of induced gene expression to regulate IL-12 after DNA transfer to mouse liver. Administration of doxycycline (Dox) enhanced IL-12 expression in a dose-dependent manner, whereas it was undetectable in serum in the noninduced state. Gene activation could be repeated several times, and sustained levels of IL-12 were achieved by daily administration of Dox. The antitumor effect of IL-12 was evaluated in a mouse model of metastatic colon cancer to the liver. Complete eradication of liver metastasis and prolonged survival was observed in all mice receiving Dox for 10 days. These data demonstrate the potential of a naked DNA gene therapy strategy to achieve tight control of IL-12 within the liver for the treatment of cancer.

INTRODUCTION

Interleukin 12 (IL-12) is a potent cytokine endowed with strong antitumor properties but considerable toxicity (1–3). In fact, although significant control of tumor growth has been achieved by systemic administration of IL-12, clinical trials were interrupted because of fatal adverse effects (2). The antitumor activity of IL-12 is mediated by stimulation of interferon γ (IFN-γ), activation of T-lymphocytes and natural killer cells, and inhibition of angiogenesis (4–6). In animal models of hepatic tumors, intratumor administration of first generation adenoviral vectors expressing IL-12 can efficiently eradicate the neoplasm (6, 7). This strategy, however, has demonstrated little efficacy in pilot clinical trials in patients with advanced primary and metastatic liver cancer, seemingly as a result of low transduction efficiency and very short duration of gene expression (8). The transfer of vectors encoding IL-12 to liver cells may have the advantage of generating high intrahepatic levels of IL-12 with lower systemic concentrations, thus increasing the therapeutic properties of the cytokine while lessening unwanted side effects. It is necessary, therefore, to develop vectors enabling tight regulation of IL-12 for its production at adequate levels and duration.

Pharmacological control of gene expression can be achieved by using the so-called "on/off" regulatory systems. Most of them are composed of two expression units: (*a*) one bearing the gene of interest under the control of an inducible promoter; and (*b*) the other carrying a constitutively expressed chimeric *trans*-activator protein able to bind a specific drug and mediate the activation or repression of the inducible promoter activity. Depending on the nature of the inducer drug, five systems adequate for their use in mammalians have been described to date: the Tet on/off (9, 10); the Pip on/off (11); and the antiprogestin-dependent (12), ecdysone-dependent (13, 14), and rapamycin-dependent (15, 16) gene switch systems. Among them, the Tet-on system exhibits the best features for applications in patients because: (*a*) the inducer doxycycline (Dox) is well tolerated in humans and has been widely used as an antibiotic; (*b*) Dox is liposoluble and has considerable tissue penetration; (*c*) it can be given orally and permits rapid gene induction/silencing switch *in vivo* in a dose-dependent manner (17); (*d*) this system has been studied in the context of numerous viral and nonviral vectors to regulate expression of various genes, and results are well documented (17–23); and (*e*) the level of gene expression in individual cells correlates directly with the dose of inducer, allowing a graded transcriptional response (24). Despite these advantages, a main drawback of this system is its relatively high basal expression. This feature would limit its use for regulation of therapeutic proteins, which are potentially toxic.

In the Tet-on system, the reverse repressor of tetracycline operon (rtetR) was fused to the herpes simplex virus VP16 transcriptional factor to generate the reverse tetracycline-controlled *trans*-activator (rtTA), which interacts with the inducible promoter in the presence of tetracycline (or analogues as Dox) and activates transcription (10). The inducible promoter is composed of seven copies of the Tet operator *tetO* fused to a cytomegalovirus minimal promoter region (CMVm). To reduce the basal expression, two kinds of chimeric repressors were developed: (*a*) one is the bacterial tetR protein fused to the repressor domain (KRAB) of the human Kox-1 protein (25); and (*b*) the other is the tetR fused to the human Mad-1 domain, which is involved in the recruitment of mSin3-histone deacetylase complex (26). Both of these repressors interact with the inducible promoter in the absence of Dox and actively silence the basal expression in culture cells and *in vivo* (26–28). Additionally, modifications on the original rtTA were made. One of the mutants, named rtTA2s-M2, binds with much lower efficiency to the *tetO* regions than rtTA in the noninduced state, and its VP16 domain was shortened to avoid cell toxicity (23, 29, 30). Moreover, rtTA2s-M2 shows higher sensitivity to Dox than rtTA, and it is able to induce the same expression levels with 10 times less inductor dose (29). Best results for gene regulation *in vivo* were obtained when combined repressor and modified *trans*-activator proteins together (28, 31).

In this study, we aimed at: (*a*) reducing the basal activity of the Tet-on system by making additional modifications without using repressor proteins; (*b*) constructing a liver-specific single vector carrying the necessary elements of the regulatory system in a short DNA

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Requests for reprints: M. Gabriela Kramer, University of Navarra-CIMA. Department of Internal Medicine, Pamplona, Spain. Fax: (34) 948-425700; E-mail: mgkramer@unav.es.

sequence with minimal leakage and high level of inducible gene expression; and (*c*) using this vector to control expression of IL-12 in the liver and analyzing its antitumoral activity in animal models of metastatic liver cancer.

MATERIALS AND METHODS

Animal and Cell Lines. Immunocompetent 6–8-week-old female BALB/c were purchased from Harlan (Barcelona, Spain). The experiments were performed in accordance with the institutional ethical commission. Human hepatoblastoma HepG2, hepatitis B virus-infected hepatocarcinoma Hep3B, cervical cancer HeLa, and adenoviral E1-transformed embryonic kidney 293 (HEK293) cells were obtained from American Type Culture Collection. CT26 is a murine cell line derived from an undifferentiated colorectal adenocarcinoma established from an *N*-nitroso-*N*-methylurethan-induced tumor (32). All cell types were cultured in DMEM supplemented with 10% fetal bovine serum (Life Technologies, Inc.) as described (7).

Plasmid Constructions. pTRE-luc was generated by inserting the luciferase gene from pGL3 basic (Promega) into *Hind*III-*Xba*I site of pTRE2 (Clontech). CMVm promoter (positions 319–438 in pTRE2) was substituted by Palb and Pcore by *Sma*I-*Hind*III fragment replacement. Palbm was generated by PCR amplification of a region lacking the 112 5′-end of Palb using forward primer: GAGGTAACCCGGGTTAATGATCTACA that has a *Sma*I site and following the same conditions as described (33). rtTA2s-M2 sequence was isolated from pUHrT62–1 (29) with *Bam*HI-*Eco*RI and placed under the control of EIIPa1AT (L1), EalbPa1AT (L2), and Phpx (L3) promoters. To construct single vectors carrying two genes oriented in opposite direction, the *Xho*I-*Xba*I 2,203-bp fragment containing the *tetO*$_7$ (position 7–318 in pTRE2)-Palb-luciferase cassette was excised from pO$_7$Palb-luc and ligated into the unique *Sal*I site of plasmids L1-rtM2, L2-rtM2, and L3-rtM2. To construct the tandem-oriented single plasmids, the *Xho*I-*Ase*I 3,379-bp fragment was excised from pO$_7$Palb-luc and ligated in L2-rtM2 linearized with *Mlu*I to generate pTonL2(T)-luc. The other two plasmids were generated from the previous one by excision of the *Not*I-*Mlu*I 3,568-bp Tet-inducible cassette and subcloning into L1-rtM2 or L3-rtM2 plasmids, digested with same enzymes. The hIL-12- and mIL-12-containing vectors were obtained by exchange of luciferase gene in pTonL2(T)-luc using *Hind*III and partial digestion with *Xba*I. The mouse IL-12-coding sequence was obtained from pBS/IL-12 (34) by *Xho*I-*Spe*I digestion. The p35 and p40 subunits of hIL-12 were obtained by PCR of cDNA from lipopolysaccharide (10 μg/ml)-activated leukocytes. Primers used were: CTGCAGACCATGGGTCCAGCGCGCAGCCTCCT and CTGCAGTTAGGAAGCATTCAGATAGCTCGTCA for p35; and CCATGGGTCACCAGCAGTTGGTCAT and GATATCTAACTGCAGGGCACAGAT for p40. The 675- (*p35*) and 994-bp (*p40*) genes were linked into pBSKII(+) (Stratagene) with internal ribosomal entry site (IRES) from pCITE-1 (Novagen). The *Spe*I-*Xho*I p35-IRES-p40 fragment was subcloned in pTonL2(T)-luc as indicated for mIL-12. Sequencing of DNA was routinely used for cloning verification.

Cell Transfections and Luciferase Detection. Cells growing in 12-well dishes to 50–60% confluence were transiently transfected by the calcium phosphate precipitation method. Plasmids were purified with Concert Nucleid Acid Purification System (Life Technologies, Inc.), and an equimolar amount of DNA corresponding to 1 μg of pTRE-luc was used. When single and separate plasmids were used in the same experiment, pBSKII(+) was included to adjust the amount of DNA. Always, 10 ng of pRL-SV40 (Promega) were added to each reaction to monitor transfection efficiency. Post-transfection culture medium (16 h) was replaced with DMEM 10% fetal bovine serum with or without Dox (Clontech). Cells were recovered at 48 h, washed twice with PBS, and lysed in 250 μl of Passive Lysis Buffer (Promega). Firefly and renilla luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). Protein concentration was calculated using the Bio-Rad protein assay. Experiments were repeated at least three times. For *in vitro* mIL-12 and hIL-12 measurement in supernatants, cell transfection was done with 2 μg of pTonL2(T)-hIL12 and pTonL2(T)-mIL12 plasmids in six-wells plates. Western blot analysis to detect luciferase protein was performed exactly as described previously (33) using goat antifirefly luciferase polyclonal antibody (Promega).

Animal Manipulation and Model of Liver Metastasis. Each plasmid DNA (20 μg) resuspended in 1.6 ml of saline was injected into mice using the hydrodynamics-based procedure (35). Dox was administrated i.p dissolved in 200 μl of saline. Blood samples were obtained by retro-orbital bleeding, and serum was recovered by centrifugation at 10,000 rpm for 10 min and stored at −20°C until protein measurement. For the liver cancer model, 150,000 CT26 cells in 50 μl of saline were inoculated in two different areas of the main liver lobe. Mice were laparotomyzed under anesthesia, and tumor growth was monitored by measurement of two perpendicular diameters using a precision caliper. Survival was checked daily, and animals were euthanazed only if moribund.

Determination of IL-12 and Alanine Aminotransferase (ALT) Levels. OptE1A human IL-12 (p70) and OptE1A mouse IL-12 (p70) ELISA kits (both from BD Bioscience PharMingen, San Diego, CA) were used for hIL-12 and mIL-12 determination. The limit of detection was 5 and 50 pg/ml, respectively. Values of mIL-12 obtained with PharMingen kit are usually higher than those obtained with another commercial kit from Endogen. ALT was measured using a Hitachi 911 Automatic Analyzer.

Statistical Analyses. All analyses were done using SPSS version 9.0 software (Chicago, IL) with *P*s of <0.05 considered to be statistically significant. The test used in each case is specified in the figure legends.

RESULTS

Reduction of Basal Expression of the Tet-On System in the Absence of Repressors. Because basal expression of genes regulated by the Tet-on system is in part the result of residual affinity of rtTA to the inducible promoter in the absence of Dox, we have used its newer version, rtTA2s-M2 (referred here as rtM2; Ref. 29). To avoid remaining leakage without using repressor molecules, we have modified the Tet-responsive promoter, based on previous data indicating that its CMVm region was active by itself (18, 33, 36) and would contribute to the basal function of Tet systems (37, 38). Therefore, we selected three notably weak liver-specific promoters consisting of a 196- and 84-bp-long mouse albumin 5′-gene region (Palb and Palbm, respectively), and the 29-bp promoter of the human hepatitis B virus core protein (Pcore), to substitute CMVm in pTRE-luc vector, which carries the luciferase reporter under the control of the Tet-inducible promoter (Fig. 1*A*). The resulting plasmids pO$_7$Palb-luc, pO$_7$Palbm-luc, and pO$_7$Pcore-luc, together with pTRE-luc, were used to transfect well-differentiated hepatic (Hep3B and HepG2) and nonhepatic (HeLa and 293) cells. In the context of the *tetO* sequences, the activity of the three liver-specific promoters (Palb, Palbm, and Pcore) was very low in all these cells. On the contrary, expression from pTRE-luc was higher in the hepatic cell lines as well as in 293 cells (Fig. 1*B*). Among the liver-specific promoters, the activity of Palb was the lowest, even in the presence of the *trans*-activator (data not shown); therefore, we selected the pO$_7$Palb-luc vector to check inducibility of gene expression and compare it with the original pTRE-luc.

A plasmid containing the *rtM2* gene driven by the CMV promoter (29) was cotransfected with either pO$_7$Palb-luc or pTRE-luc in HepG2 and Hep3B cells. The amount of luciferase (Fig. 1*C*) and its activity (Fig. 1*D*) were measured in the absence and presence of *trans*-activator and Dox. The results showed that expression from pTRE-luc, which was already detectable on a Western blot in HepG2 cells (Fig. 1*C*), was enhanced ~70 times when cotransfected with CMV-rtM2 in Hep3B (Fig. 1*D*). An additional 6.5-fold induction of luciferase expression was achieved with a saturating dose of Dox. On the other hand, expression from pO$_7$Palb promoter was undetectable when analyzed by Western blot, even in the presence of rtM2, and the protein could only be detected after addition of Dox (Fig. 1*C*). Here, the luciferase activity slightly increased because of the *trans*-activator, and a 50-fold induction was obtained with Dox (Fig. 1*D*). Final protein levels were higher when directed with pTRE-luc, although the highest fold induction was achieved with pO$_7$Palb-luc. This fact indicates that by reducing the basal expression of the system, a decrease in the final induced protein concentration will also occur.

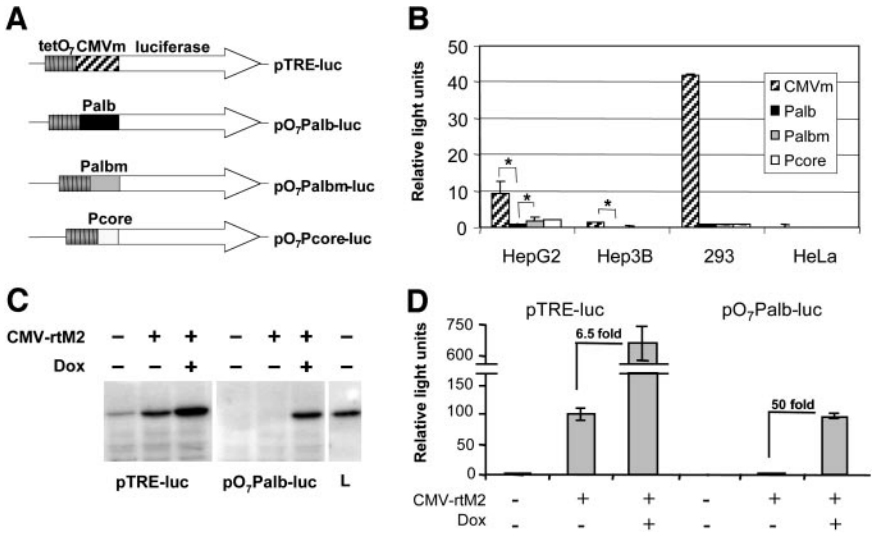

Fig. 1. Effect of minimal promoter substitution in basal and induced gene expression. *A*, schematic representation of plasmids containing the firefly luciferase reporter gene. The 119-bp cytomegalovirus minimal promoter (CMVm) of the original Tet-on system present in pTRE2-luc was substituted by the 29-bp hepatitis B virus core protein promoter (*Pcore*), the 196-bp mouse albumin gene promoter (*Palb*), or its 84-bp 3′-region (*Palbm*). The location of the seven operator copies (*tetO*$_7$) from *Escherichia coli* tetracycline operon is shown. *B*, hepatic and nonhepatic human cells were cotransfected with 1 μg of each of the plasmids mentioned above and 10 ng of an SV40-renilla luciferase plasmid as transfection control. The ratio between firefly and renilla luciferase light units is represented. CMVm, Palb, Palbm, and Pcore refer to the sequence present in each inducible promoter. $*$, $P < 0.05$, Mann-Whitney *U* test. *C*, luciferase protein detected in HepG2 lysates. Cells were transfected with the indicated plasmids and treated (+) or not (−) with 1 μg/ml Dox. Because efficiency of transfection was almost identical, Western blot analysis was performed with 10 μg of total cell lysate protein of each preparation. L refers to recombinant luciferase (2 ng) used as a positive control. *D*, luciferase activity was measured in Hep3B lysates after cotransfection with the indicated plasmids with or without Dox.

Construction of Single Vectors for Liver-Specific Regulable Gene Expression. To restrict expression to hepatocytes, we used a liver-specific promoter to direct rtM2. Because the amount of *trans*-activator produced can affect the leakiness of the system (39), we tested different promoters with graded activity to select the sequence, allowing more inducibility while keeping minimal basal expression. Taking advantage of our chimeric promoters characterized previously (33), we have chosen three of them, named EIIPa1AT, EalbPa1AT (human α-1 antitrypsin promoter fused to the hepatitis B virus EII enhancer or mouse albumin distal sequence, respectively), and Phpx (promoter of human hemopexin gene). EIIPa1AT (here, L1) is two times stronger than CMV in HepG2 cells, followed by EalbPa1AT (here, L2), with 70% of CMV activity and Phpx (here, L3) with <1% of CMV function (33).

Because the relative orientation of the expression cassettes can influence gene regulation and leakage of the Tet-on system (23, 28), we constructed single vectors carrying both transcription units (L1, L2, or L3-rtM2 and pO$_7$Palb-luc) in two different orientations: tandem or opposite (Fig. 2*A*). Fold induction of luciferase activity in the tandem/opposite constructs was ~20/30, 35/40, and 1000/500 for the L1-, L2-, and L3-containing plasmids, respectively (Fig. 2*B*). Similar results were obtained in Hep3B cells (data not shown). These data showed that: (*a*) the basal, as well as induced expression, are directly proportional to the strength of the promoter used to direct rtM2 expression; (*b*) when both units are organized in tandem, gene expression was higher than when opposite oriented; and (*c*) fold induction was inversely proportional to the basal expression (maximal fold induction with L3-containing plasmids).

To analyze liver specificity of the single constructs, we transfected 293 cells with each of the six plasmids, and luciferase was measured in the presence of Dox. The final protein concentration was ~10 times lower compared with the induced protein in hepatic cells (Fig. 2, *B* and *C*). Taken into account that an ubiquitous promoter like CMV is ≥10 times more active in 293 than HepG2 cells (33), we conclude that these plasmids are strongly attenuated in nonhepatic cells. To compare activity of the original and modified Tet-responsive promoters in these cells, we performed equimolar cotransfections with L1-rtM2 and pTRE-luc or pO$_7$Palb-luc. About 40 times lower expression levels (with Dox) were obtained with pO$_7$Palb-luc compared with the original pTRE-luc plasmid (Fig. 2*C*). The low but still sizable reporter expression achieved in 293 cells may be attributable to residual activity of L1, L2, and L3 promoters in nonliver cells (33). These data strengthen the potential of the Tet-on system to respond to extremely low concentrations of the *trans*-activator, indicating the high sensitivity of the system.

Regulation of IL-12 and Dox Dose Dependence Study *in Vivo*. Among all vectors (Fig. 2*A*), we have selected pTonL2(T)-luc to control IL-12 expression, because: (*a*) the induced expression levels were high, whereas basal activity was acceptable; and (*b*) L2 promoter allows a more stable gene expression in the liver compared with L1 and L3 (33). Thus, we have substituted the luciferase gene in pTonL2(T)-luc by the coding sequences of the human and murine IL-12, resulting in plasmids pTonL2(T)-hIL12 and pTonL2(T)-mIL12, respectively. The response of both vectors to increasing doses of Dox was studied in HepG2 cells. Our results demonstrate that the system responds to very low doses of Dox (0.1 ng/ml) and that saturation takes place between 0.1 and 1 μg/ml (data not shown). Human IL-12 is devoid of most of its known biological functions in rodents (40), and in accordance to that, we were unable to detect IFN-γ, a mayor mediator of IL-12 activity (41) in mice treated with hIL-12 (data not shown). Therefore, we used this protein to study the regulation and kinetics of the inducible system. To this end, we transferred the pTonL2(T)-hIL12 vector to the liver of BALB/c mice using the hydrodynamics-based procedure (35, 42). Fifteen days after plasmid injection, different doses of Dox were given by i.p. route. No cytokine was detected in serum in the absence of the drug. With the lowest Dox dose tested (0.5 mg/kg), hIL-12 levels were ~100 pg/ml, and the maximum levels (2800–3500 pg/ml) were achieved with 5 and 50 mg/kg, respectively (Fig. 3).

***In Vivo* Pharmacokinetics of hIL-12 Induction.** To analyze the latency of hIL-12 activation/inactivation and long-term behavior of the modified Tet-on system, we transferred the pTonL2(T)-hIL12 plasmid to mice, and one dose of 50 mg/kg Dox was then administrated every 10–25 days for a period of 75 days. Last induction was done 6 months after plasmid infusion. Serum hIL-12 was measured in individual animals at different time points. A peak of hIL-12 expression occurred 10 h after induction with Dox and decreased to undetectable levels in 24–48 h (Fig. 4*A*). Spaced Dox administration resulted in a correspondent hIL-12 induction with similar peak values and same kinetics as the first one until day 75. However, induced hIL-12 levels substantially decreased when analyzed 6 months after plasmid administration (Fig. 4*A*).

Strategies to treat liver cancer by intrahepatic IL-12 expression will seemingly need continuous synthesis of the cytokine for a prolonged period of time. To investigate whether persistent levels of IL-12 could be achieved, we injected Dox every 12 or 24 h to a separate group of

animals that had received the plasmid 3 months before. Blood samples were taken, and hIL-12 was measured 10 h after Dox administration. Using both protocols, similar continuous amounts of the cytokine were detected in serum, although hIL-12 expression was more stable when Dox was repeated every 12 h (Fig. 4*B*).

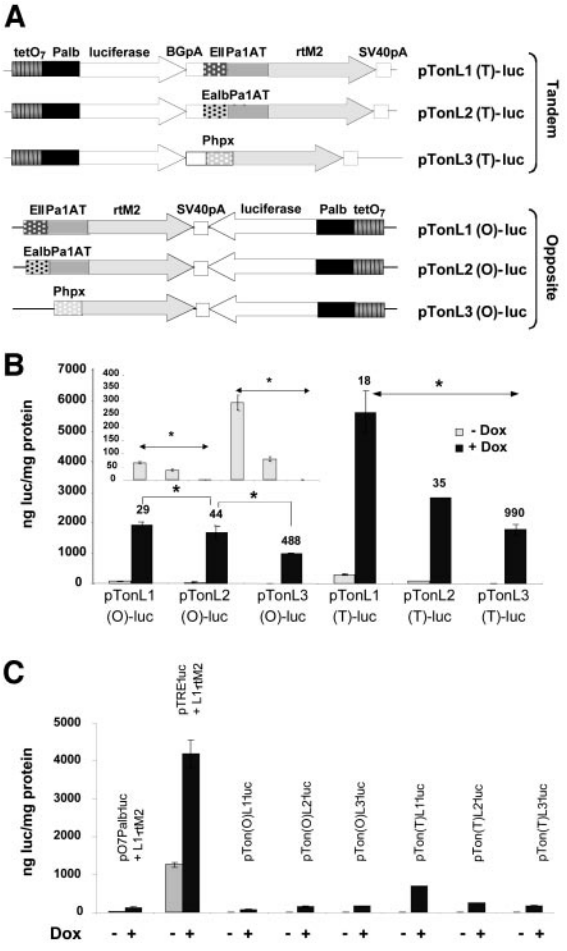

Fig. 2. Effect of gene disposition in single vectors. *A*, schematic structure of single plasmid vectors. *Arrows*, direction of transcription for luciferase and rtM2 genes. Chimeric liver-specific promoters (670–200 pb) are described in "Results" (33). Inducible promoter consists of *tetO* operators (311 bp) fused to the 196-bp mouse albumin promoter (*Palb*). BGpA (1167 bp) contains the rabbit β globin poli(A), and SV40pA (221 bp) has the bi-directional viral poli(A) signal. *B*, the indicated plasmids were transfected in HepG2 cells, and luciferase levels were measured in lysates of untreated (−) or Dox (1 μg/ml)-treated (+) samples, using a recombinant luciferase standard. Fold induction is indicated at the *top* of each column. An amplification of the noninduced expression in the same experiment is shown in the *small rectangle*. Significative differences between groups are indicated. *, $P < 0.05$, Dunnet *C* test (↔). *, $P < 0.05$, Mann-Whitney *U* test (—). *C*, pTRE2-luc and pO7Palb-luc plasmids were cotransfected with L1-rtM2 (~2 μg total) in 293 cells. Single plasmids were cotranfected with pBSKII(+) to adjust the same amount of DNA.

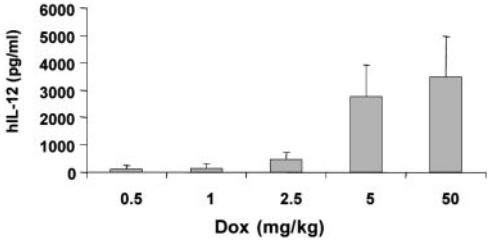

Fig. 3. *In vivo* Dox dose-dependent *IL*-12 expression. pTonL2(T)-hIL12 was administered to BALB/c mice by hydrodynamics-injection. Fifteen days later, increasing doses of Dox were injected i.p to five different groups of mice ($n = 4$). Serum samples were collected 10 h after induction, and hIL-12 was quantified by ELISA. The mean value and SD are represented for each group of animals.

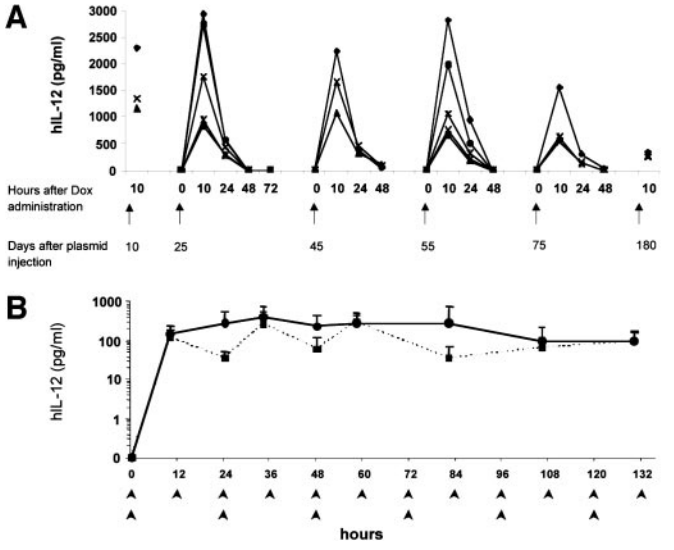

Fig. 4. Kinetics of IL-12 induction. *A*, pTonL2(T)-hIL12 was injected into a group of mice ($n = 5$), and 50 mg/kg Dox were administrated once every 10–25 days (*arrows*). At days 10, 45, and 180, only three animals were induced. Serum samples were collected at the indicated time points for individual hIL-12 measurement. *B*, repeated administration (*arrows*) of 5 mg/kg Dox was given to mice ($n = 6$) bearing the plasmid every 12 h (*continuous line*) or 24 h (*dotted line*), and hIL-12 was quantified 10 h after Dox injection. Human IL-12 was undetectable before starting the drug treatment.

Efficacy of mIL-12 Expression in the Liver for the Treatment of Liver Cancer. To analyze the antitumor effect of IL-12 expressed in the liver parenchyma, we first developed a mouse model of multifocal liver metastasis. We established that 150,000 colon cancer CT26 cells injected in syngenic BALB/c mouse liver were able to develop a tumor of ~200 mm^3 in 7 days (Fig. 5*A*). Thus, we used these conditions in our subsequent studies. Plasmids pTonL2(T)-mIL12, pTonL2(T)-luc, or vehicle (saline) were administrated 1 week before injection of the tumor cells (to escape the initial period postvector injection that causes noninduced expression of mIL-12 attributable to hydrodynamic liver stress.[4] One week after tumor implantation, engraftment of cancer cells was checked by laparotomy, and six groups were established: (*a*) saline (no plasmid); (*b*) Luc-D50:pTonL2(T)-luc + 50 mg/kg Dox; (*c*) IL12–Dox:pTonL2(T)-mIL12 without Dox; (*d*) IL12-D2.5:pTonL2(T)-mIL12 + 2.5 mg/kg Dox; and (*e*) IL12-D50:pTonL2(T)-mIL12 + 50 mg/kg Dox. Administration of Dox was performed every 24 h for 10 days. Sixteen days after initiation of Dox treatment, mice were laparotomized to evaluate the progression of tumor growth. All animals that had received pTonL2(T)-mIL12 plus Dox showed no evidence of tumor (Fig. 5, *A* and *B*) and long-term survival (Fig. 5*C*). On the contrary, animals given saline or Luc-D50 developed big tumors, and all animals died (or were euthanized) between days 37 and 52 after tumor implantation. Interestingly, four of nine animals from the IL12-Dox group rejected the tumor and survived the entire duration of the study. Serum levels of mIL-12 in this group were undetectable, as well as groups Saline and Luc-D50. Only groups IL12-D2.5 and IL12-D50 expressed the cytokine, which reached ~350 and 1400 ng/ml at day 5, respectively (Fig. 6*A*). To evaluate the liver toxicity of the therapy, ALT levels were measured in the same serum samples. As shown in Fig. 6*B*, mice treated with pTonL2(T)-mIL12 exhibited a slight increase of ALT only when administrated 2.5 or 50 mg/kg Dox. Control animals, as well as IL12-Dox, showed no significant differences in ALT levels.

[4] Unpublished results.

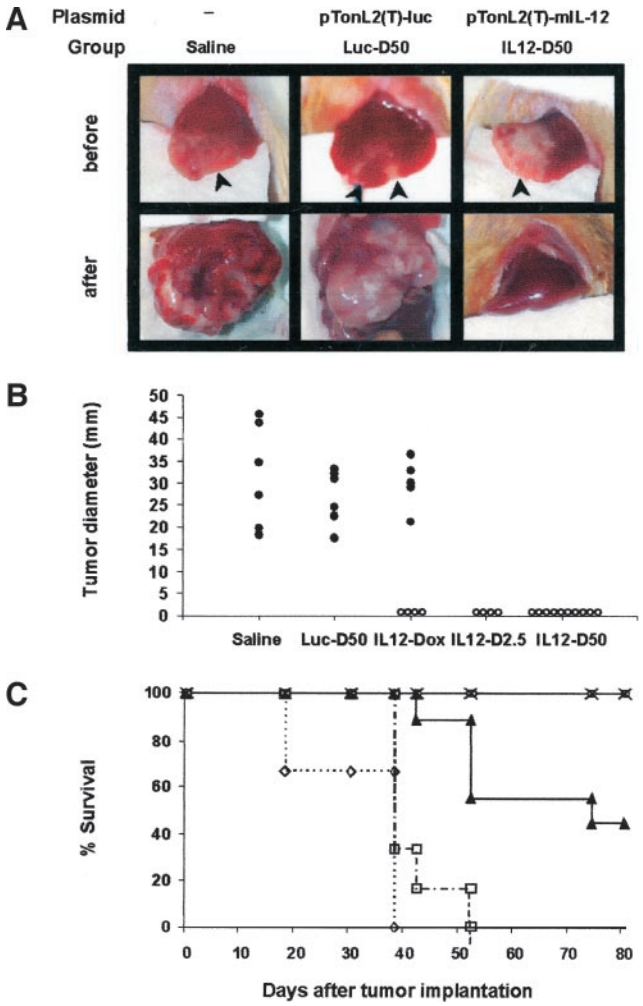

Fig. 5. Antitumor activity of intrahepatic interleukin-12 expression. *A*, pictures of the liver from representative mice obtained after laparotomy. The indicated plasmids were transferred to mice 1 week before CT26 cell implantation. Seven days later, tumor engraftment was confirmed (*before*), and then Dox was administrated every 24 h for 10 days to the indicated groups. Tumor growth was checked 6 days later (*after*). Saline group did not receive plasmid. Luc-D50 and IL12-D50 were treated with 50 mg/kg Dox. *Arrows*, tumors. *B*, tumor diameter of individual mice is represented by *circles*: ●, animals that died or were sacrificed when moribund; ○, animals survived after 80 days of follow-up. IL12-Dox received pTonL2(T)-mIL12 plasmid, and no Dox. IL12-D2.5 group was treated with 2.5 mg/kg Dox. The rest of the groups were the same as in *A*. *C*, survival curve. Groups represented are Saline (·◇·), Luc-D50 (– · –), IL12-Dox (–▲–), IL12-D2.5 (–×–), and IL12-D50 (-○-).

DISCUSSION

The use of cytokines with strong antitumor effect for the treatment of malignancies like liver cancer is hampered by the systemic toxicity of these agents (43). Therefore, in this study, we pursued two main aims: (*a*) the construction of a vector with improved Tet-on system to allow tight regulation of gene expression in the liver; and (*b*) application of such a vector encoding IL-12 to treat hepatic tumors.

The Tet-on system is composed of a transcriptional activator that binds to a minimal promoter in the presence of Dox and induces gene expression. Although this system has great potential for gene therapy purposes, its widespread use is limited by its relatively high basal activity. Two improved *trans*-activator proteins, rtTA2s-S2 and rtTA2s-M2, were recently developed to generate a system displaying lower background activity and higher window of Dox-dependent gene induction as compared with the original rtTA activator (23, 29, 31). Among them, rtTA2s-M2 (rtM2) exerted better characteristics regarding Dox sensitivity and inducibility, although this protein still needs to be combined with a repressor protein (tTS) to minimize gene expression in the noninduced state (28, 31). When including repressor-coding sequences, the resulting increase of the DNA size of all of the system would limit its use in vectors with restricted cloning capacity. Moreover, the presence of a constitutive repressor protein could affect cellular functions by interacting with endogenous factors (25). For these reasons, we attempted to modify the system to reduce basal expression in the liver avoiding repressor molecules.

To this aim, we have changed elements in the Tet-responsive promoter because we found that its intrinsic activity greatly contributed to leakiness of the system. In fact, the substantial activity in 293 cells of its CMVm region, which contains the TATA box and also several binding sites for cellular transcriptional factors (9, 44), seemed to be responsible for the difficulties in generating adenoviral vectors carrying a proapoptotic factor regulated with the Tet-off system (45). Thus, by simply substituting CMVm by a liver-specific, but very weak, mouse albumin promoter (Palb), we found a 35–50 times reduction in basal expression in human cell lines (Figs. 1 and 2). A shorter version of the albumin promoter of 84 bp lacking the 5′-region showed higher activity in the same cell lines, indicating a possible role in promoter suppression of the deleted region.

With the aim of generating a single vector, six different constructs were made using three liver-specific promoters with graded activity (33). The functional analysis of these vectors showed a direct correlation between the strength of the promoter that directs rtM2 and the level of noninduced and induced gene expression. In addition, we observed that basal expression correlated directly with the maximal induced protein level until saturation of the system. When the transcriptional units were oriented in the opposite orientation, lower reporter protein was obtained (as compared with tandem-oriented cassettes), probably by reduction of gene expression through transcriptional interference.

On the basis of the above data, we constructed a single vector with two expression units placed in tandem: (*a*) one for liver-specific expression of rtM2 using the L2 promoter; and (*b*) the other with the O_7Palb promoter to control IL-12 synthesis. We saw no basal expression and a clear dose-dependent induction with Dox, which could be maintained for >2 months without any important reduction in the level of hIL-12. Moreover, continuous administration of the inducer for several days resulted in sustained serum levels of the protein

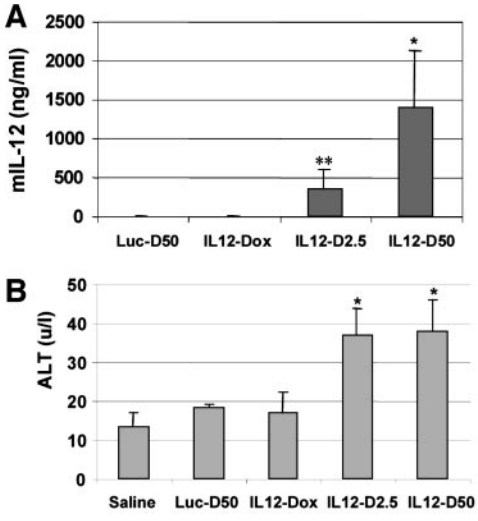

Fig. 6. IL-12 expression and liver toxicity during therapy. *A*, serum levels of mIL12 were measured in control mice (*Saline* and *IL12-Dox*) and 5 days after administration of 2.5 (*IL12-D2.5*) and 50 (*IL12-D50*) mg/kg Dox. The values are given as the mean of each group ± SD. *, $P < 0.05$; **, $P < 0.01$, Mann-Whitney *U* test. *B*, alanine aminotransferase (*ALT*) levels were measured in the same serum samples. *, $P < 0.05$, Dunnet *C* test.

during all this period. Administration of Dox after 3 months of plasmid injection resulted in a substantial reduction of inducible gene expression. This effect may be related to promoter silencing, loss of DNA by degradation, or simply turnover of transfected cells. The long-term persistence of gene products regulated by Tet systems in immunocompetent mice indicates that the immune response against the *trans*-activator is not the cause for declining gene expression with time (17, 18, 20, 23, 31, 39).

Using a murine model of metastatic colon cancer to the liver (a condition which in humans lacks curative therapy), we found a complete eradication of tumors in animals treated with TonL2(T)-mIL12 plus Dox (either at low or high doses) for 10 days, whereas all animals that received saline or similar vector-encoding luciferase showed progressive tumor growth and death before 2 months. The antitumor treatment occurred with acceptable toxicity because the rise of transaminases was mild, and all those mice showed long-term survival. Interestingly, four of nine animals which received TonL2(T)-mIL12 but no Dox also eliminated the tumor, suggesting that noninduced expression of mIL-12 within the liver at very low levels (not detectable in serum) does not prevent tumor engraftment but may be sufficient to activate a delayed antitumor immune response in some animals.

In conclusion, we have modified the Tet-on system by reducing the basal expression without adding additional coding elements and maintaining high inducibility in liver cells. On the basis of these modifications, we have constructed a single vector to express IL-12 within the liver. This vector enabled tight regulation of the cytokine and proved to be very efficient as tool for therapy of liver cancer. Although the plasmid here described could be used in the context of long-term expression viral vectors, progress in techniques for delivery of naked DNA to the human liver may facilitate the clinical use of these constructs, considering their easier high-scale production and better acceptance by regulatory agencies than the currently used viral vectors.

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REFERENCES

1. Orange JS, Wolf SF, Biron CA. Effects of IL-12 on the response and susceptibility to experimental viral infections. J Immunol 1994;152:1253–64.
2. Leonard JP, Sherman ML, Fisher GL, et al. Effects of single-dose interleukin-12 exposure on interleukin-12-associated toxicity and interferon-γ production. Blood 1997;90:2541–8.
3. Lui VW, Falo LD Jr, Huang L. Systemic production of IL-12 by naked DNA mediated gene transfer: toxicity and attenuation of transgene expression in vivo. J Gene Med 2001;3:384–93.
4. Tannenbaum CS, Wicker N, Armstrong D, et al. Cytokine and chemokine expression in tumors of mice receiving systemic therapy with IL-12. J Immunol 1996;156:693–9.
5. Kodama T, Takeda K, Shimozato O, et al. Perforin-dependent NK cell cytotoxicity is sufficient for anti-metastatic effect of IL-12. Eur J Immunol 1999;29:1390–6.
6. Barajas M, Mazzolini G, Genove G, et al. Gene therapy of orthotopic hepatocellular carcinoma in rats using adenovirus coding for interleukin 12. Hepatology 2001;33:52–61.
7. Drozdzik M, Qian C, Xie X, et al. Combined gene therapy with suicide gene and interleukin-12 is more efficient than therapy with one gene alone in a murine model of hepatocellular carcinoma. J Hepatol 2000;32:279–86.
8. Sangro B, Mazzolini G, Ruiz J, et al. A Phase I clinical trial of intratumoral injection of adenovirus encoding interleukin-12 in patients with hepatocellular carcinoma and other advanced gastrointestinal tumors. J Clin Oncol. In press 2004.
9. Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci USA 1992;89:5547–51.
10. Gossen M, Freundlieb S, Bender G, Muller G, Hillen W, Bujard H. Transcriptional activation by tetracyclines in mammalian cells. Science 1995;268:1766–9.
11. Fussenegger M, Morris RP, Fux C, et al. Streptogramin-based gene regulation systems for mammalian cells. Nat Biotechnol 2000;18:1203–8.
12. Wang Y, DeMayo FJ, Tsai SY, O'Malley BW. Ligand-inducible and liver-specific target gene expression in transgenic mice. Nat Biotechnol 1997;15:239–43.
13. Christopherson KS, Mark MR, Bajaj V, Godowski PJ. Ecdysteroid-dependent regulation of genes in mammalian cells by a Drosophila ecdysone receptor and chimeric transactivators. Proc Natl Acad Sci USA 1992;89:6314–8.
14. No D, Yao TP, Evans RM. Ecdysone-inducible gene expression in mammalian cells and transgenic mice. Proc Natl Acad Sci USA 1996;93:3346–51.
15. Rivera VM, Clackson T, Natesan S, et al. A humanized system for pharmacologic control of gene expression. Nat Med 1996;2:1028–32.
16. Liberles SD, Diver ST, Austin DJ, Schreiber SL. Inducible gene expression and protein translocation using nontoxic ligands identified by a mammalian three-hybrid screen. Proc Natl Acad Sci USA 1997;94:7825–30.
17. Aurisicchio L, Bujard H, Hillen W, et al. Regulated and prolonged expression of mIFN(α) in immunocompetent mice mediated by a helper-dependent adenovirus vector. Gene Ther 2001;8:1817–25.
18. Bohl D, Salvetti A, Moullier P, Heard JM. Control of erythropoietin delivery by doxycycline in mice after intramuscular injection of adeno-associated vector. Blood 1998;92:1512–7.
19. Kafri T, van Praag H, Gage FH, Verma IM. Lentiviral vectors: regulated gene expression. Mol Ther 2000;1:516–21.
20. Fitzsimons HL, McKenzie JM, During MJ. Insulators coupled to a minimal bidirectional tet cassette for tight regulation of rAAV-mediated gene transfer in the mammalian brain. Gene Ther 2001;8:1675–81.
21. Rubinchik S, Wang D, Yu H, et al. A complex adenovirus vector that delivers FASL-GFP with combined prostate-specific and tetracycline-regulated expression. Mol Ther 2001;4:416–26.
22. Unsinger J, Kroger A, Hauser H, Wirth D. Retroviral vectors for the transduction of autoregulated, bidirectional expression cassettes. Mol Ther 2001;4:484–9.
23. Lamartina S, Roscilli G, Rinaudo CD, et al. Stringent control of gene expression in vivo by using novel doxycycline-dependent trans-activators. Hum Gene Ther 2002; 13:199–210.
24. Kringstein AM, Rossi FM, Hofmann A, Blau HM. Graded transcriptional response to different concentrations of a single transactivator. Proc Natl Acad Sci USA 1998;95: 13670–5.
25. Deuschle U, Meyer WK, Thiesen HJ. Tetracycline-reversible silencing of eukaryotic promoters. Mol Cell Biol 1995;15:1907–14.
26. Jiang W, Zhou L, Breyer B, et al. Tetracycline-regulated gene expression mediated by a novel chimeric repressor that recruits histone deacetylases in mammalian cells. J Biol Chem 2001;276:45168–74.
27. Forster K, Helbl V, Lederer T, Urlinger S, Wittenburg N, Hillen W. Tetracycline-inducible expression systems with reduced basal activity in mammalian cells. Nucleic Acids Res 1999;27:708–10.
28. Salucci V, Scarito A, Aurisicchio L, et al. Tight control of gene expression by a helper-dependent adenovirus vector carrying the rtTA2(s)-M2 tetracycline transactivator and repressor system. Gene Ther 2002;9:1415–21.
29. Urlinger S, Baron U, Thellmann M, Hasan MT, Bujard H, Hillen W. Exploring the sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and sensitivity. Proc Natl Acad Sci USA 2000;97:7963–8.
30. Baron U, Gossen M, Bujard H. Tetracycline-controlled transcription in eukaryotes: novel transactivators with graded transactivation potential. Nucleic Acids Res 1997;25:2723–9.
31. Lamartina S, Silvi L, Roscilli G, et al. Construction of an rtTA2(s)-m2/tts(kid)-based transcription regulatory switch that displays no basal activity, good inducibility, and high responsiveness to doxycycline in mice and non-human primates. Mol Ther 2003;7:271–80.
32. Brattain MG, Strobel-Stevens J, Fine D, Webb M, Sarrif AM. Establishment of mouse colonic carcinoma cell lines with different metastatic properties. Cancer Res 1980; 40:2142–6.
33. Kramer MG, Barajas M, Razquin N, et al. In vitro and in vivo comparative study of chimeric liver-specific promoters. Mol Ther 2003;7:375–85.
34. Sun Y, Qian C, Peng D, Prieto J. Gene transfer to liver cancer cells of B7–1 plus interleukin 12 changes immunoeffector mechanisms and suppresses helper T cell type 1 cytokine production induced by interleukin 12 alone. Hum Gene Ther 2000;11:127–38.
35. Zhang G, Budker V, Wolff JA. High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. Hum Gene Ther 1999;10:1735–7.
36. Ackland-Berglund CE, Leib DA. Efficacy of tetracycline-controlled gene expression is influenced by cell type. Biotechniques 1995;18:196–200.
37. Baron U, Bujard H. Tet repressor-based system for regulated gene expression in eukaryotic cells: principles and advances. Methods Enzymol 2000;327:401–21.
38. Hoffmann A, Villalba M, Journot L, Spengler D. A novel tetracycline-dependent expression vector with low basal expression and potent regulatory properties in various mammalian cell lines. Nucleic Acids Res 1997;25:1078–9.
39. McGee Sanftner LH, Rendahl KG, Quiroz D, et al. Recombinant AAV-mediated delivery of a tet-inducible reporter gene to the rat retina. Mol Ther 2001;3:688–96.
40. Schoenhaut DS, Chua AO, Wolitzky AG, et al. Cloning and expression of murine IL-12. J Immunol 1992;148:3433–40.
41. Brunda MJ, Luistro L, Hendrzak JA, Fountoulakis M, Garotta G, Gately MK. Role of interferon-gamma in mediating the antitumor efficacy of interleukin-12. J Immunother Emphasis Tumor Immunol 1995;17:71–7.
42. Liu F, Song Y, Liu D. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. Gene Ther 1999;6:1258–66.
43. Mazzolini G, Prieto J, Melero I. Gene therapy of cancer with interleukin-12. Curr Pharm Des 2003;9:1981–91.
44. Boshart M, Weber F, Jahn G, Dorsch-Hasler K, Fleckenstein B, Schaffner W. A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. Cell 1985;41:521–30.
45. Rubinchik S, Ding R, Qiu AJ, Zhang F, Dong J. Adenoviral vector which delivers FasL-GFP fusion protein regulated by the tet-inducible expression system. Gene Ther 2000;7:875–85.