In vivo manipulation of interleukin-2 expression by a retroviral tetracycline (tet)-regulated system

Claudia Pitzer,[1] Katharina Schindowski,[1] Sigmund Pomer,[2] Thomas Wirth,[3] and Margot Zöller[1,4]

[1]Department of Tumor Progression and Immune Defense, German Cancer Research Center, Heidelberg, Germany; [2]Department of Urology, University of Heidelberg, Heidelberg, Germany; [3]Institute for Medical Radiology and Cell Research, Würzburg, Germany; and [4]Department of Applied Genetics, University of Karlsruhe, Karlsruhe, Germany.

We have used the tetracycline (tet)-regulated system as described previously to evaluate the applicability of controlled gene expression in cancer gene therapy. As a model gene, we used the human *interleukin-2* (*IL-2*) gene, which has been placed under the transcriptional control of the tetO/promoter. Human melanoma cells were transduced by two modified retroviral tet vectors containing the transactivator regulatory unit and the *IL-2* gene driven by the tetO/promoter, respectively. In the absence of tet, IL-2 expression in the target cells was stable over several months. IL-2 production was in the range of 40 U/10^6 cells/24 hours. A fine tuning of IL-2 expression could be achieved by culturing the transduced cells with increasing doses of tet, whereby a concentration of 500 ng/mL tet in the culture medium abrogated IL-2 expression. Most importantly for clinical application, IL-2 expression by the transduced melanoma cells could also be regulated *in vivo*. When nu/nu mice were inoculated with the transduced tumor cells, they failed to develop tumors. Instead, the inhibition of IL-2 expression in the transduced tumor cells by oral administration of tet led to subcutaneous tumor growth; this growth rate was comparable with the growth rate of subcutaneously inoculated untransduced parental cells. The finding demonstrates the applicability of the tet-regulated system in cancer gene therapy.

Key words: Tetracycline; interleukin-2 gene; gene therapy; tumor vaccines.

A high level of expression and the controllable delivery of gene products constitute two important parameters to accomplish effective gene therapy. The choice of an adequate expression vector system could be an important tool to assess the optimal expression of the target gene. A system that answers these questions has been described recently.[1] Target genes are placed under the control of regulatory sequences (tetO) from the tetracycline (tet)-resistance operon of transposon 10. The tet-regulated system is composed of two elements, each of which is carried on a separate plasmid vector. One component, the regulator unit, encodes the transactivator (tTA). tTA is a fusion protein composed of the tet repressor of *Escherichia coli* and the transcriptional activation domain of the VP16 protein of the herpes simplex virus. The second component, the response unit, is composed of the *E. coli*-derived tet resistance operon regulatory element (tetO) embedded within a minimal cytomegalovirus (CMV) promoter. Expression of a target gene inserted downstream of the tetO/minimal CMV promoter is dependent upon the tTA, which binds tetO sequences. In the presence of the antibiotic, the binding of the tTA to the tetO promoter is impaired, resulting in shutoff of target gene expression in cultured cells[1–6] and transgenic mice.[7–10] In the absence of the antibiotic, expression of the target gene is induced.

The tet-regulated system allows not only a qualitative off-on transition but also a fine tuning of gene expression with different tet concentrations.[1,11,12] Regulation is restricted to the target gene, and host genes are not affected. The required tet concentrations seem nontoxic.[1]

We have used a retroviral modification of the tet-regulated system to evaluate the feasibility of controlled gene expression in tumor therapy. As a model system, we manipulated the expression of the human *interleukin-2* (*IL-2*) gene in the human melanoma cell line MeWo. In the animal model, we could demonstrate the efficacy of IL-2 expression in mounting an antitumor response of tumor cells expressing IL-2, which has been described repeatedly.[13–17] In the absence of tet, the expression of IL-2 by the tumor cells prevented subcutaneous (s.c.) growth of the melanoma cells in nu/nu mice. Instead, tumor growth was unimpaired when IL-2 expression was abolished by a supply of the antibiotic in the drinking water. This finding demonstrates for the first time the applicability of the retroviral tet-regulated

Received January 12, 1998; accepted June 27, 1998.

Address correspondence and reprints to Dr. Margot Zöller, Department of Tumor Progression and Immune Defense, German Cancer Research Center, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany.

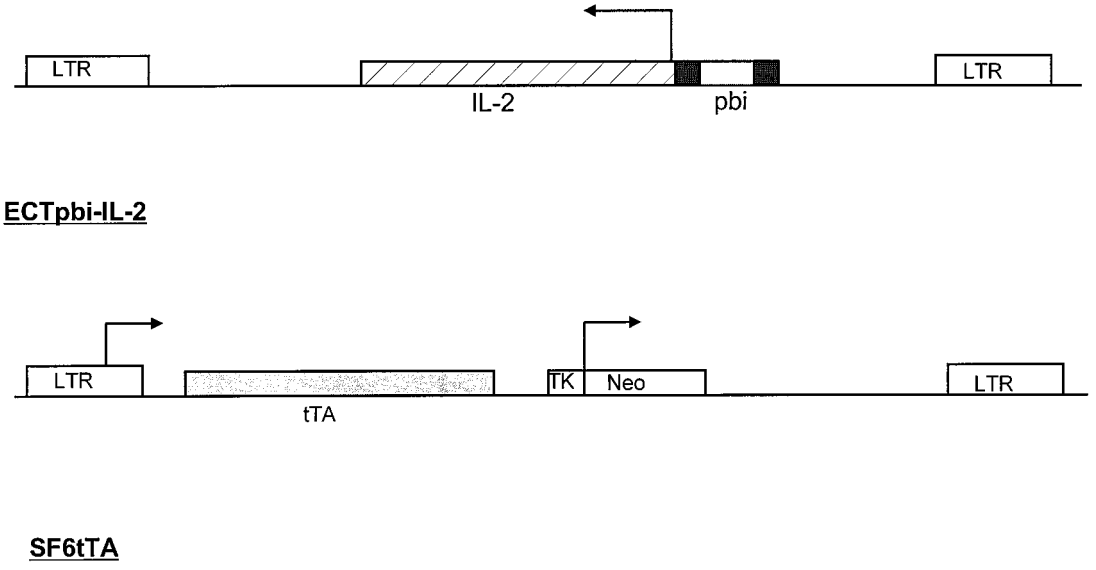

Figure 1. Schematic representation of tet-regulatable retroviral vectors. SF6tTA contains the transactivator regulator unit tTA, which is under the transcriptional control of the 5′ retroviral LTR. The neomycin resistance gene (*Neo*) is under the transcriptional control of the internal thymidine kinase promoter. ECT2pbiIL-2 contains the human *IL-2* gene, which is controlled by the tetO/promoter. The tetO sequences are flanked by two divergently orientated minimal CMV promoters (bidirectional promoter, pbi). tTA, transactivator; TK, herpes simplex virus type 1 thymidine kinase promoter.

system in cancer gene therapy. The model creates prospects for *in vivo* manipulation of immunologically and therapeutically relevant genes.

MATERIALS AND METHODS

Cell culture

The retroviral packaging cell lines Bosc23 and PA317, NIH3T3 (mouse embryo fibroblasts), HeLa (human cervix carcinoma cell line), and CTLL-2 (cytotoxic T-lymphocyte line 2) were purchased from the American Type Culture Collection (Manassas, Va). The human melanoma cell line MeWo has been described previously by Bean et al.[18] All cell lines were maintained at 37°C in 5% CO_2 and Dulbecco's modified Eagle's medium (Life Technologies, Eggenstein, Germany) supplemented with 10% fetal bovine sera (Sigma, Deisenhofen, Germany), 1% glutamine, and 1% penicillin-streptomycin (Life Technologies). CTLL-2 cells were cultured in RPMI 1640 medium (Life Technologies) containing 10% fetal bovine sera and 20 international units/mL human recombinant IL-2 (rIL-2) (Chiron, Emeryville, Calif).

Retroviral vector constructs, viral production, and transduction

The retroviral vector constructs are schematically illustrated in Figure 1. SF6tTA and ECT2pbiIL-2 are based on the retroviral vector SFG (kindly provided by R. Mulligan, Harvard Medical School, Boston, Mass). The plasmid SF6tTA was constructed by inserting the transactivator regulator element tTA from pUHD15-1[1] and the neomycin resistance marker gene. ECT2pbiIL-2 contains the human *IL-2* gene, which is controlled by the tetO/promoter. In this construct tetO is flanked by two divergently orientated CMV minimal promoters (the bidirectional promoter, pbi[11]). The human *IL-2* gene and its tetO/promoter are located in antisense orientation relative to the 5′ long terminal repeat (LTR), so that the human *IL-2* gene is driven only by the tetO/promoter.

For packaging of the constructed vectors, 10 μg of each plasmid DNA was transfected into the helper-free ecotropic packaging cell line Bosc23 by calcium phosphate precipitation as described previously.[19] The virus supernatant of Bosc23 was harvested after 48 hours, passed through a 0.45-μm filter, adjusted to 8 μg/mL polybrene, and used to transduce the amphotropic packaging cell line PA317.

The PA317 cells transduced with SF6tTA were selected for G418 resistance at a concentration of 400 μg/mL. Individual colonies were analyzed for tTA expression using reverse transcriptase-polymerase chain reaction (RT-PCR). Viral titers were tested by their capacity to confer G418 resistance to NIH3T3 cells. The viral supernatant of the producer colony that showed the highest titer (2×10^6 colony-forming units/mL, data not shown) and tTA expression was used to generate MeWo/tTA by the infection of parental MeWo cells. The producer cell line PA317 was transduced with ECT2pbiIL-2, and the virus-containing supernatant was used to infect a clonal population of MeWo/tTA. Because a second selectable marker gene was absent on the ECTpbiIL-2 plasmid, clonal isolates of double transductants were generated by single-cell plating on a 96-well plate. The resulting double transductant MeWo-tTA/IL-2 was cultured in the absence and presence of tet (500 ng/mL) in the culture medium.

Transfection and luciferase assay

NIH3T3 and HeLa cells (5×10^5) were first transfected with 1 μg of the luciferase reporter plasmid pUHD131-1 by calcium phosphate precipitation.[20] At 1 day after transfection, the medium was changed. On the following day, cells were infected with SF6tTA virus supernatant from the Bosc23 packaging cell line (NIH3T3 target cells) or supernatant from the PA317 supernatant (HeLa target cells). At 2 days after infection, cells

were harvested for the luciferase assay. Cells were washed in phosphate-buffered saline and subsequently lysed in luciferase extraction buffer (0.1 M potassium phosphate, 2 mM ethylenediaminetetraacetic acid, 0.7 mM Triton X-100, and 1 mM dithiothreitol) at 4°C. Cell lysates were collected from the plate using a cell scraper and centrifuged. Aliquots (30 μL) of the supernatant were mixed with 370 μL of luciferase reaction buffer containing 25 mM glycylglycine, 1 mM adenosine triphosphate, and 10 mM $MgSO_4$ and assayed for luciferase activity in a Lumat (Berthold, Wildbad, Germany). D-luciferin was used at 0.5 mM. Luciferase activity was adjusted to the protein content of the lysates using the Bradford assay (Bio-Rad, Munich, Germany).

Determination of IL-2 production by enzyme-linked immunosorbent assay (ELISA)

Supernatants were collected from MeWo-tTA/IL-2 cells seeded at a density of 2×10^5. Culture supernatant was assayed in triplicate for IL-2 production by ELISA. Briefly, 96-well plates coated with an anti-human IL-2 monoclonal antibody were incubated for 1 hour at 37°C with the test samples. After three washes, the plates were incubated for 1 hour with horseradish peroxidase-conjugated goat anti-human IL-2 according to the manufacturer's protocol. The enzymatic reaction was developed with a chromagen substrate, and optical density was measured on an ELISA plate reader.

Determination of IL-2 production by the CTLL-2 bioassay

The biological activity of IL-2 released by the transduced cells was tested with an IL-2-dependent CTLL proliferation assay. Briefly, dilutions of MeWo-tTA/IL-2 culture supernatant (treated with tet or left untreated) were transferred to a 96-well culture plate containing 3×10^4 CTLL-2 cells per well. CTLL-2 cells cultured in RPMI 1640 medium or in a known amount of IL-2 were used as negative and positive controls, respectively. After 6 hours, 0.5 μCi of [^{3}H]thymidine was added to each well, and the culture was incubated for an additional 24 hours. Cells were harvested with a cell harvester (Pharmacia, Uppsala, Sweden), and [^{3}H]thymidine incorporation was counted in a liquid scintillation counter (LKB, Turku, Finland). A standard IL-2 curve containing known concentrations permitted the quantitation of IL-2 levels.

IL-2 and tTA expression by RT-PCR

Producer cell lines and the double transductant MeWo-tTA/IL-2 were tested for tTA and IL-2 gene expression. Total RNA was extracted from 2×10^5 cells according to Chomczynski and Sacchi.[21] A total of 2 μg of RNA was reverse transcribed to cDNA using the standard protocol.[20] cDNA was subjected to PCR amplification. Reactions were performed in 50-μL reaction mixtures that contained 50 mM KCl, 20 mM tris(hydroxymethyl)aminomethane-HCl, 1.5 mM $MgCl_2$, 15 mM deoxynucleoside triphosphate, 25 pmol of each primer, the template DNA, and 2 U of *Taq* polymerase. The thermal cycler profile was as follows: 30 cycles of 60 seconds for denaturing at 94°C and 30 seconds for annealing/elongation at 50°C for the IL-2 primers (R&D Systems, Minneapolis, Minn). For tTA PCR, annealing was performed at 58°C for 45 seconds, and elongation was accomplished at 64°C for 1 minute in the 40-cycle profile. The amplified products were electrophoresed in a 1.5% agarose gel.

Provirus integration by genomic PCR

Genomic DNA was isolated by cell lysis in 1% sodium dodecyl sulfate, 200 μL proteinase K, 100 mM NaCl, 10 mM tris(hydroxymethyl)aminomethane, and 25 mM ethylenediaminetetraacetic acid at 55°C for 16 hours after DNA isolation; isolation was accomplished by phenol-chloroform extraction and ethanol precipitation.[20] A total of 1 μg of DNA was amplified by PCR using tTA and the *IL-2* gene-specific primers according to the same protocol as described above.

Tumorigenicity in nu/nu mice

Parental MeWo cells and MeWo-tTA/IL-2 cells (previously cultured in the presence and absence of tet) (5×10^6) were procured from tissue culture flasks, washed, and injected s.c. into the flanks of congenitally athymic nude mice. The group of mice injected with tet-pretreated MeWo-tTA/IL-2 received 200 μg/mL tet dissolved in 5% sucrose supplied in the drinking water, which was exchanged every 3 days. Bidirectional tumor diameters were measured using a caliper at regular intervals for 60 days.

Cytotoxicity assay

The natural killer (NK) activity of spleen cells from nu/nu mice was assayed on MeWo target cells in a 4-hour ^{51}Cr release assay. For preactivation, spleen cells (3×10^6) were cultured for 6 days in the presence and the absence of tet together with 3×10^5 irradiated (100 Gy) parental MeWo cells, IL-2-transduced MeWo cells, and parental MeWo cells, for which the culture medium contained 20 U of rIL-2.

Target cells were prepared by culturing cells for 2 hours in the presence of 100 μCi of ^{51}Cr. A total of 10^4 labeled target cells per well were mixed with titrated amounts of effector cells to yield effector to target ratios of 50–6:1 and were incubated for 4 hours. Plates were centrifuged, and released radioactivity was measured in 100-μL aliquots of the supernatants. Maximum release was determined by incubating the target cells with 2% sodium dodecyl sulfate; spontaneous release was measured by incubating the target cells with the culture medium.

RESULTS

Tet-dependent regulation of luciferase activity with the retroviral tTA transactivator construct SF6tTA

To test the activity of the retroviral transactivator construct SF6tTA in the tet-dependent regulation system, we used NIH3T3 and HeLa cells as target cells. At 2 days before infection, target cells were transiently transfected with the luciferase reporter plasmid pUHD131-1 by calcium precipitation. NIH3T3 and HeLa cells were then infected with the SF6tTA virus supernatant from the Bosc23 and PA317 packaging cells, respectively, in the presence (500 ng/mL) and absence of tet. The luciferase activity of the pooled cells was determined 2 days thereafter. In the absence of tet, we observed an elevation of luciferase activity of up to ~500-fold in both cell lines (Table 1). This observation indicated that the tTA gene driven by the internal 5′ LTR promoter was active. Furthermore, the tet-inducible system, including the retroviral SF6tTA regulator unit in cooperation with a tet promoter response unit, seemed tightly regulated.

Table 1. Tet-Dependent Regulation of Luciferase Activity with the Retroviral Transactivator Construct SF6tTA

	Luciferase activity (rlu†/mg of total protein)		
	With tet	Without tet	Activation factor
NIH3T3*	540496	611	884
HeLa	436689	655	667

* NIH3T3 and HeLa cells were transiently transfected with the luciferase reporter plasmid pUHD131-1 and infected with the SF6tTA virus supernatant from the ecotropic Bosc23 (for NIH3T3 cells) or the amphotropic PA317 (for HeLa cells) packaging cells. Luciferase activity in the absence and the presence (500 ng/mL) of tet reflects the inducible regulation. Values are the arithmetic means of three independent luciferase determinations (in triplicate).

† rlu, relative light units.

Retroviral gene transfer and generation of MeWo cells expressing IL-2 and tTA simultaneously

The retroviral vector constructs that were used to introduce and express the tTA and the human IL-2 gene simultaneously in the MeWo cell line are shown above (Fig 1). As described in *Materials and Methods*, we first generated packaging cell lines that produce tTA and IL-2 virus, respectively. The supernatants of the producer cell lines were tested for viral titer and gene expression either by RT-PCR analysis (tTA) or by ELISA and the CTLL-2 bioassay (IL-2). The colonies showing the highest viral titer and gene expression were used to infect the target MeWo cell line. MeWo cells were infected first with the tTA containing virus supernatant. Neomycin-resistant colonies were selected and cloned. Thereafter, tTA^+ clones were infected with *IL-2*-containing supernatant.

Clonal isolates of the double transductant MeWo-*tTA/IL-2* clones were analyzed for proviral DNA amplification and gene expression. Amplification of the respective proviral *IL-2* and *tTA* genes is shown for one clone by PCR of genomic DNA samples and by RT-PCR for proviral mRNA (Fig 2). Untransduced MeWo cells did not express the *tTA* gene or the *IL-2* gene (data not shown).

Tet-regulated expression of IL-2 and dose-dependent expression of IL-2 at different tet concentrations

Several MeWo-tTA/IL-2 clones were cultured in the absence of tet, and IL-2 production was determined by ELISA and by CTLL-2 assay. The amount of IL-2 produced by the different MeWo-tTA/IL-2 clones varied from 5 to 40 U/10^6 cells/24 hours (data not shown). The clone that expressed the highest amount of IL-2 (40 U) was selected for subsequent studies. Transfected MeWo-tTA/IL-2 cells were then cultured in the presence of increasing doses of tet, and the expression of IL-2 was analyzed by RT-PCR and ELISA. Figure 3, A and B, shows a gradual reduction of IL-2 expression with an increasing dose of tet. The data indicated that IL-2 expression in the transduced tumor cells could be reduced gradually by increasing concentrations of tet; a complete inhibition of IL-2 expression could be achieved by 100 ng/mL tet.

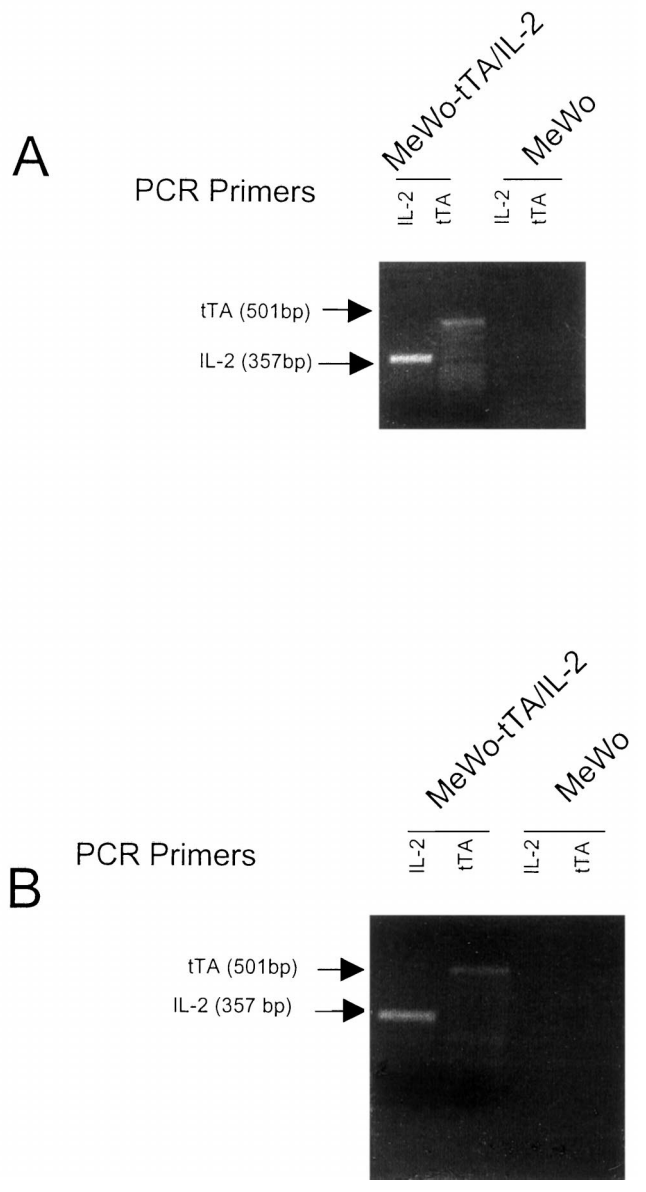

Figure 2. Provirus integration and expression shown by DNA **(A)** and RT-PCR **(B)**. DNA and RNA were isolated from the transduced MeWo-tTA/IL-2 cells and the parental MeWo cells and were amplified using tTA and IL-2-specific primers. Amplified products were separated on a 1.5% agarose gel stained with ethidium bromide.

Exposure to tet interferes with activation of NK cells by IL-2-transduced tumor cells

As shown in Table 2 NK/lymphokine activated killer (LAK) activity was significantly increased after the coculture of spleen cells with the transduced MeWo-tTA/IL-2. By adding tet to the culture medium, spleen cells did not become activated and displayed cytotoxicity values comparable with those obtained after coculture

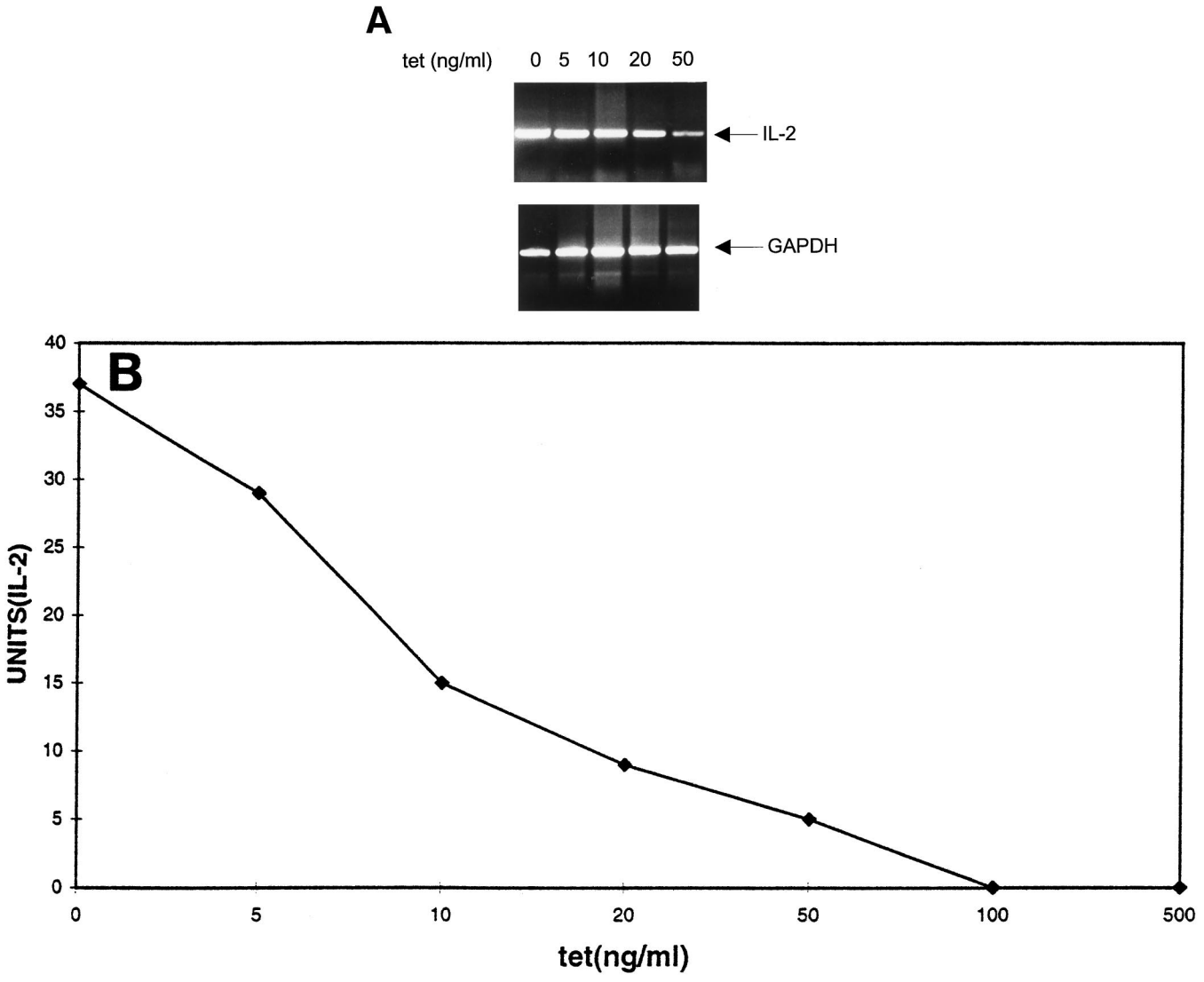

Figure 3. Expression of IL-2 decreases with increasing doses of tet. Results of RT-PCR **(A)** and CTLL-2 **(B)** bioassays are shown. **A:** RNA from MeWo-tTA/IL-2 cells was isolated and reverse transcribed; the cDNA was amplified using specific primers for the respective genes IL-2 and glyceraldehyde-3-phosphate dehydrogenase. Amplified products were separated on a 1% agarose gel stained with ethidium bromide. **B:** IL-2 production was determined with the CTLL-2 bioassay. IL-2 levels were evaluated as U/10^6 cells/24 hours.

with untransduced MeWo cells. However, tet as such did not influence the activation of NK cells, because NK cells became activated to the same degree in cultures containing exogenous IL-2 or exogenous IL-2 plus tet. This finding demonstrated that the secreted IL-2 is biologically active and that the supply of tet has no toxic effects on NK/LAK cells.

Tumorigenicity in nude mice

Finally, we evaluated the *in vivo* applicability of the tet system in tumor-bearing mice. It is known that a s.c. injection of 5×10^6 parental MeWo cells leads to tumor growth in 100% of nu/nu mice.[22] In the reported experiment, nu/nu mice received a s.c. inoculation of 5×10^6 transduced and nontransduced MeWo cells. One group of mice inoculated with transduced MeWo-tTA/IL-2

Table 2. Cytotoxic Activity of NK Cells Against MeWo Cells

Cell line*	% Specific lysis†
MeWo	10.3
MeWo + tet	10.7
MeWo + rIL-2	55.6
MeWo + rIL-2 + tet	53.9
MeWo-tTA/IL-2	60.0
MeWo-tTA/IL-2 + tet	10.3

* Spleen cells from nu/nu mice were cultured for 6 days with irradiated parental untransduced MeWo cells (in the absence or presence of tet and in the absence or presence of exogenous IL-2; rIL-2) and transduced MeWo cells (MeWo-tTA/IL-2 in the absence or presence of tet).

† Cytotoxicity against ^{51}Cr target MeWo cells in a 4-hour release assay is shown (effector to target ratio = 25:1).

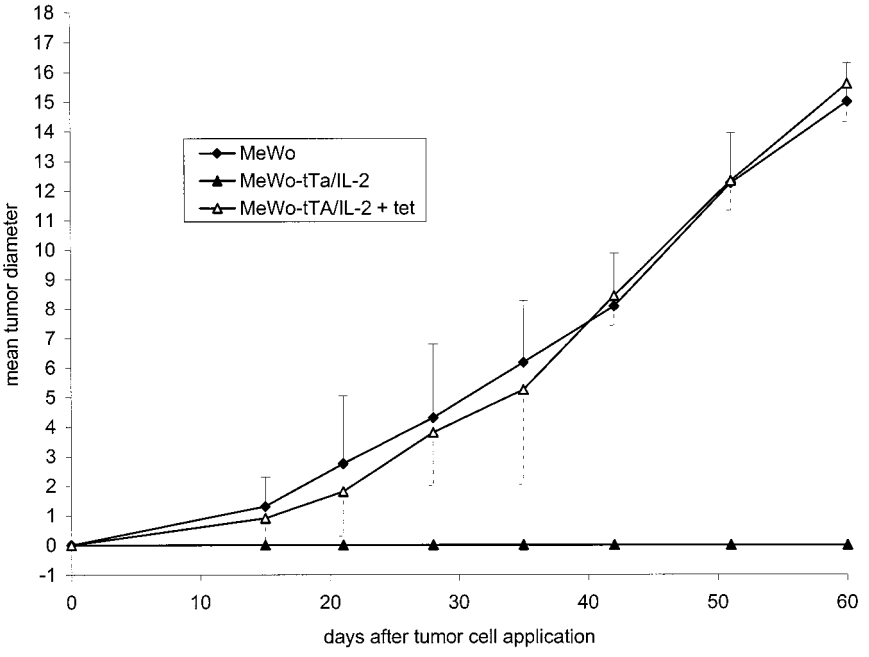

Figure 4. Tumorigenicity of transduced MeWo-tTA/IL-2 cells and *in vivo* inhibition of IL-2 expression by a tet exposure. Groups of six mice in each category were injected with 5×10^6 cells as indicated. MeWo-tTA/IL-2 plus tet mice received 200 μg/mL tet in drinking water. The mean diameters $\pm$ SD of the s.c. growing tumors are shown. Animals were killed after 60 days. Macroscopic and histological evaluations at the implantation site and of different organs such as the liver and lung did not reveal any sign of tumor growth in the MeWo-tTA/IL-2-treated mice.

cells received tet in drinking water, whereas the other group received water without the antibiotic. No tumor growth was observed in the group of animals receiving the transduced MeWo-tTA/IL-2, which underlines the efficacy of the antitumor immune response induced by tumor cells expressing IL-2. The growth rate of MeWo-tTA/IL-2 when mice received tet in drinking water did not significantly differ from the growth rate of parental MeWo cells (Fig 4). This result demonstrates that IL-2 production in the injected tumor cells can be switched off *in vivo* by an oral administration of tet. This finding is particularly worthwhile, considering that the tumor cells were implanted into the subcutis, which is not an easily accessible region.

DISCUSSION

The feasibility of regulating the expression of biologically active substances such as hormones, cytokines, or chemokines could be of great clinical importance in tumor therapy, but also in autoimmune diseases and chronic infections. To explore the possibility of *in vivo*-regulated expression of the cytokine IL-2, we have used the classical two-plasmid tet system as delineated by Gossen and Bujard[1] and modified it as a retroviral system to transduce the tTA gene and the human IL-2 gene simultaneously in the human melanoma cell line MeWo. We could demonstrate regulation of IL-2 expression *in vitro* as well as *in vivo*. In the latter situation, IL-2 expression could be regulated systemically (i.e., in a s.c. growing tumor by oral application of tet). In agreement with previous results, we observed neither *in vitro* nor *in vivo* leakiness of the binary system.[1,2,11]

To test the functionality of the new constructed retroviral transactivator construct SF6tTA, we transiently transfected NIH3T3 and HeLa cells. Because there is no retroviral reporter construct available, we had to use the original nonretroviral luciferase reporter plasmid, in which the luciferase reporter gene is placed downstream of the chimeric tetO/CMV minimal promoter. We also had to transiently transfect it before the target cells were infected with the SF6tTA-containing supernatant. The determination of the luciferase activity in the absence of tet revealed up to a 500-fold induction, which was tightly regulated. Because the reporter plasmid was not of a retroviral origin, the induction rate as well as the tight regulation cannot be directly transmitted to the retroviral system. Nonetheless, transient transfection with the retroviral transactivator construct SF6tTA allowed us to prove the functionality of the retroviral tTA vector construct.

When generating the MeWo-tTA/IL-2 cells, we noted that distinct clones produced variable amounts of IL-2, whereby it should be noted that the amount of IL-2 production was a stable feature of the individual clones. All functional studies were pursued with a clone that produced 40 U/10^6 cells/24 hours. This dose has been reported by several groups to be the appropriate range to induce an antitumor immune response.[16,23] The efficiency of IL-2 production by MeWo-tTA/IL-2 to initiate the activation of NK cells was proven *in vitro* as well as *in vivo*. Spleen cells of nu/nu mice exhibited a significantly higher cytotoxic potential against MeWo cells after coculture with transduced MeWo-tTA/IL-2 than after coculture with untransduced MeWo cells. Although a similar increase in cytotoxic activity could be achieved by the addition of exogenous IL-2, the cyto

toxic activity of spleen cells cultured with MeWo-tTA/IL-2 in the presence of tet displayed only background cytolytic activity. The fact that the addition of tet to cultures containing exogenous IL-2 did not influence LAK activation proves that the applied concentration of tet was not toxic. *In vivo*, the amount of IL-2 expressed by the transduced MeWo cells was sufficient to completely prevent tumor growth, whereas an application of tet in drinking water was sufficient to fully restore tumorigenicity (i.e., in the presence of tet, the transduced MeWo showed a comparable growth rate with untransduced MeWo cells). As demonstrated *in vitro* and controlled *in vivo* (data not shown), tet had no affect on NK/LAK activity. Thus, the restoration of tumor growth cannot be assigned to an impaired immune defense, but most likely will be attributable to the switch-off of IL-2 production. It is already known that a supply of tet in drinking water leads to a continuous dissemination of the antibiotic in different tissues, whereby tet is readily absorbed and broadly distributed without toxicity at the concentration needed to regulate the activity of the promoter.[10] This was demonstrated for vascularized tissues such as the liver in transgenic mice.[10] We have proven that the antibiotic also easily accesses nonvascularized tissue, such as a depot of tumor cells in a s.c. pocket. This assumption derives from the observation that the effect of inhibiting IL-2 expression was readily visible already at the start of tumor growth (i.e., at 10 days after application of the tumor cells and tet, respectively).

Thus, although the *in vivo* applicability of the tet system has already been shown using transgenic expression systems in animals carrying the tTA and the gene of interest[7–10] or by directly injecting the gene into the tissue,[24,25] we have demonstrated for the first time the *in vivo* feasibility of the retroviral tet-regulated system in gene therapy with IL-2 transduced tumor cells in the mouse.

Independent of the discussion for the need for different retroviral expression vectors or promoters to achieve optimal expression of the gene of interest, the described *in vivo* manipulation of gene expression in transduced tumor cells with the tet-regulated system provides perspectives on gene therapy. The off-on regulation and, even more significant, the fine tuning, could be of advantage in the expression of certain genes for which efficacy is tightly linked to an optimal level of gene expression. Beyond this, the use of a bidirectional promoter[11] allows for the possibility of two different genes being distinctly coregulated.

ACKNOWLEDGMENTS

We thank Drs. S. Freundlieb, U. Baron, and H. Bujard for supplying plasmids and for helpful discussions. This work was funded by a program project grant from the Bundesministerium für Forschung und Technologie (to M.Z. and S.P.).

REFERENCES

1. Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci USA.* 1992;89:5547–5551.
2. Damke H, Gossen M, Freundlieb S, et al. Tightly regulated and inducible expression of dominant interfering dynamin mutant in stably transformed HeLa cells. *Methods Enzymol.* 1995;257:209–220.
3. Wang L, Takagaki Y, Manley JL. Targeted disruption of an essential vertebrate gene: ASF/SF2 is required for cell viability. *Genes Dev.* 1996;10:2588–2599.
4. Hwang JJ, Scuric Z, Anderson F. Novel retroviral vector transferring a suicide gene and a selectable marker gene with enhanced gene expression by using a tetracycline-responsive expression system. *J Virol.* 1996;70:8138–8141.
5. Schultze N, Burki Y, Lang Y, et al. Efficient control of gene expression by single integration of the tetracycline system in transgenic mice. *Nat Biotechnol.* 1996;14:499–503.
6. Fruh K, Gossen M, Wang K, et al. Displacement of housekeeping proteosome subunits by MHC-encoded LMPs: a newly discovered mechanism for modulating the multicatalytic proteinase complex. *EMBO J.* 1994;13:3236–3244.
7. Furth PA, Onge SL, Böger H, et al. Temporal control of gene expression in transgenic mice by tetracycline-responsive promoter. *Proc Natl Acad Sci USA.* 1994;91:9302–9306.
8. Passman RS, Fishman GI. Regulated expression of foreign genes in vivo after germline transfer. *J Clin Invest.* 1994;94:2421–2425.
9. Efrat S, Fuscodemane D, Lemberg H, et al. Conditional transformation of a pancreatic β cell line derived from transgenic mice expressing a tetracycline-regulated oncogene. *Proc Natl Acad Sci USA.* 1995;92:3576–3580.
10. Kistner A, Gossen M, Zimmerman F, et al. Doxycycline-mediated quantitative and tissue-specific control of gene expression in transgenic mice. *Proc Natl Acad Sci USA.* 1996;93:10933–10938.
11. Baron U, Freundlich S, Gossen M, Bujard H. Co-regulation of two gene activities by tetracycline via a bidirectional promoter. *Nucleic Acids Res.* 1995;23:3605–3606.
12. Maxwell IH, Spitzer AL, Long CJ, et al. Autonomous parvovirus transduction of a gene under control of tissue-specific or inducible promoters. *Gene Ther.* 1996;3:28–36.
13. Gansbacher B, Zier K, Daniels B, et al. Interleukin-2 gene transfer into tumor cells abrogates tumorigenicity and induces protective immunity. *J Exp Med.* 1990;172:1217–1224.
14. Tsai S-CJ, Gansbacher B, Tait L, et al. Induction of antitumor immunity by interleukin-2 gene-transduced mouse mammary tumor cells versus transduced mammary stromal fibroblasts. *J Natl Cancer Inst.* 1993;85:546–553.
15. Zier KS, Salvadori S, Cronin KC, Gansbacher B. Vaccination with IL-2-secreting tumor cells stimulates the generation of IL-2 responsive T-cells and prevents the development of unresponsiveness. *Cancer Gene Ther.* 1994;1:43–50.
16. Fearon E, Pardoll D, Itaya T, et al. Interleukin 2 production by tumor cells bypasses T helper function in the generation of an antitumor response. *Cell.* 1990;60:397–403.
17. Abdel-Wahab Z, Li W-P, Osanto S, et al. Transduction of human melanoma cells with interleukin-2 gene reduced

tumorigenicity and enhances host antitumor immunity: a nude mouse model. *Cell Immunol.* 1994;159:26–39.
18. Bean MA, Bloom BR, Herberman RB, et al. Cell-mediated cytotoxicity for bladder carcinoma. *Cancer Res.* 1975; 35:2902–2913.
19. Pear W, Nolan GP, Scott ML, Baltimore D. Production of high-titer helper-free retroviruses by transient transfection. *Proc Natl Acad Sci USA.* 1993;90:8392–8396.
20. Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: A Laboratory Manual.* 2nd Ed. Plainview, New York: Cold-Spring Harbor Laboratory Press.
21. Chomczynski P, Sacchi N. Single-step method of RNA isolation by guanidinium thiocyanate phenol-chloroform extraction. *Anal Biochem.* 1987;162:156–159.
22. Matzku S, Schuhmacher J, Kirchgeßner H, Brüggen J. Labeling of monoclonal antibodies with a 67Ga-phenolic aminocarboxylic acid chelate. *Eur J Nucl Med.* 1986;12:405–412.
23. Fakhrai H, Shawler DL, Gjerset R, et al. Cytokine gene therapy with interleukin-2-transduced fibroblasts: effects of IL-2 dose on anti-tumor immunity. *Hum Gene Ther.* 1995;6:591–601.
24. Dhawan J, Rando TA, Elson S, et al. Tetracycline-regulated gene expression following direct gene transfer into mouse skeletal muscle. *Somat Cell Mol Genet.* 1995;21: 233–240.
25. Fishman GI, Kaplan ML, Buttrick PM. Tetracycline-regulated cardiac gene expression in vivo. *J Clin Invest.* 1994;93:1864–1868.