Thymidine kinase obliteration: Creation of transgenic mice with controlled immune deficiency

RICHARD A. HEYMAN*, EMILIANA BORRELLI*, JAYNE LESLEY†, DEBORAH ANDERSON*,
DOUGLAS D. RICHMAN‡, STEPHEN M. BAIRD‡, ROBERT HYMAN†, AND RONALD M. EVANS*

*Howard Hughes Medical Institute and Gene Expression Laboratory, †Department of Cancer Biology, The Salk Institute, and ‡Department of Pathology, Veterans Administration Medical Center, San Diego, CA 92138

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ABSTRACT The cell-specific expression of herpes simplex virus 1 thymidine kinase (HSV-1-tk) has provided a simple and highly efficient technique to achieve conditional ablation of targeted cell types in transgenic mice. The ablation is induced by treating transgenic animals expressing HSV-1-tk with the antiherpetic drug ganciclovir. In lymphoid tissues of mice expressing HSV-1-tk from an immunoglobulin promoter, administration of ganciclovir leads to massive destruction of B- and T-cell lineages. Tissues not expressing HSV-1-tk are insensitive to drug treatment. After depletion of >99% of total thymocytes, a number of progenitor cells remain that are able to repopulate all T-cell lineages within 7 days. The ability to control and direct ablation allows for creation of conditional mutant phenotypes at precise periods of development. This technique also provides a potential means to enrich stem cell populations as well as permitting the creation of animal models for particular pathological conditions.

An understanding of the self-renewing nature of stem cells and the events that trigger their differentiation represents a major challenge to developmental biology. Our laboratory and others have exploited the creation of transgenic animals as a strategy to study vertebrate development. Utilizing tissue-specific promoters and enhancers, it is possible to direct expression of cloned genes to restricted cell types enabling the experimental manipulation of cellular and organ physiology (1, 2). A reciprocal to this augmentation approach would be the creation of a transgenic hypomorph (i.e., a mouse deficient in a particular cellular function). This would allow an alternative means to evaluate the contribution of a cell or gene to a particular developmental program. To address this problem, we have developed a method for creating transgenic hypomorphs that results in the conditional ablation of specific cell types during defined periods of development and differentiation. Our strategy involves expression of an enzyme that is not itself deleterious but is capable of metabolizing a benign substrate to a toxic product.

One potential candidate for this strategy is the herpes simplex virus 1 thymidine kinase (HSV-1-tk). HSV-1-tk is by itself not harmful to mammalian cells. However, in contrast to mammalian thymidine kinase, HSV-1-tk is capable of phosphorylating specific nucleoside analogs, such as acyclovir (ACV), to the nucleoside monophosphate (3, 4). The nucleoside monophosphate is phosphorylated by cellular kinases to the nucleoside triphosphate and incorporated into DNA leading to inhibition of DNA synthesis and cell death (4, 5). The high selectivity of the viral thymidine kinase for these nucleoside analogs and the very low affinity of mammalian thymidine kinase for these nucleosides make it possible to selectively kill HSV-1-tk-expressing cells among a population of dividing cells.

We have demonstrated (6) the feasibility of this conditional-ablation approach in cell lines transfected with the gene for HSV-1-tk. The success of this approach *in vitro* suggested that it might be exploited to study cellular differentiation and lineage relationships *in vivo*. Such an approach might be accomplished by expressing HSV-1-tk under control of a cell-specific promoter likely to be active in a population of differentiating cells. We have targeted expression of HSV-1-tk to the lymphoid system by using an immunoglobulin promoter/enhancer. HSV-1-tk enzyme activity was restricted to spleen, lymph nodes, bone marrow, and thymus. In lymphoid tissues of transgenic mice that express HSV-1-tk, there is a massive depletion of B and T lymphocytes after nucleoside analog treatment. In contrast, cells not expressing HSV-1-tk are insensitive to nucleoside analog exposure. This ablation technique, referred to as thymidine kinase obliteration (TKO), allows one to control the precise timing of toxic insult, the degree of ablation, and the potential for recovery. This method should enable analysis of lineage relationships and cell function at defined periods of development.

MATERIALS AND METHODS

DNA Constructions. The construction of pKHTK has been reported (6). It contains a 682-base-pair (bp) fragment (*Xba*I–*Eco*RI) of the mouse μ heavy chain enhancer (7, 8), a 1100-bp fragment (*Eco*RI) of the mouse κ light chain promoter (9), and a 1500-bp fragment containing the coding sequence of HSV-1-tk. This construct was made by replacing the chloramphenicol acetyltransferase gene of pKCATH (10) with the HSV-1-tk gene (11). The 1.5-kilobase fragment of the HSV-1-tk gene is from position +2 relative to the transcription start site to 236 bp downstream of the polyadenylation site.

Transgenic Mice. DNA was prepared for microinjection by digestion of pKHTK with *Avr* II. This cuts 100 bp downstream of the 5' boundary of the enhancer and on the 3' side of the HSV-1-tk gene and contains no vector sequences. The fragment was injected into fertilized mouse embryos from CB6F1/J males $\times$ CB6F1/J females. The male KHTK transgenic mice had a reduced fertility rate and, therefore, pedigrees were maintained by breeding transgenic females. Tissue extracts from the transgenic mice were assayed for HSV-1-tk activity by monitoring conversion of [3 H]acyclovir to [3 H]acyclovir mono-, di-, and triphosphates, as described by Fyfe *et al.* (4).

Administration of Ganciclovir (GCV). The nucleoside analog, GCV (9-[(1,3-dihydroxy-2-propoxy)methyl] guanine), was generously provided by Ed Kirk (Burroughs Wellcome). GCV was continuously administered by using a miniosmotic pump (Alzet, model 2001, Alza) implanted subcutaneously and containing GCV at 125 mg/ml.

Flow Cytometry. Cell surface antigen expression was analyzed by flow cytometry using a modified Los Alamos flow microfluorimeter (12). For one parameter analysis of B220, Thy-1, L3T4, and Lyt-2, cells were stained with biotin-conjugated antibodies followed by fluorescein-conjugated avidin. Fluorescein-conjugated goat anti-mouse immunoglobulin was used to stain for surface immunoglobulin. Two-color flow cytometry was carried out using reagents directly conjugated with fluorescein or with biotin followed by phycoerythrin-conjugated streptavidin as described by Lesley *et al.* (12).

Inhibition of Cellular Growth by GCV in an HSV-1-tk Expressing Cell Line. We have demonstrated (6) specific cell ablation *in vitro* by showing that nucleoside analogs, acyclovir (ACV) and FIAU [1-(2-deoxy-2-fluoro- β -D-arabinofuranosyl)-5-iodouracil], selectively inhibited DNA synthesis and cell growth in cells expressing HSV-1-tk. St. Clair *et al.* (13) suggested that an analog of ACV, GCV, would be more effective than ACV in inhibiting DNA synthesis in HSV-1-tk-transformed cells. Employing the previously described experimental procedure (6), we determined the ability of GCV to selectively inhibit growth in a cell line that conditionally expresses HSV-1-tk. After a 72-hr incubation, the concentration of GCV required to achieve 50% inhibition of cell growth was 3×10^{-8} M in cells expressing HSV-1-tk and 10^{-6} M in cells not expressing HSV-1-tk. Greater than 90% of cells expressing HSV-1-tk were killed at 3×10^{-7} M.

RESULTS

Expression of an HSV-1-tk Construct in Lymphoid Tissues of Transgenic Mice. Previous studies from our laboratory have demonstrated that the expression of HSV-1-tk in cultured cells can form the basis of a conditional toxic phenotype (6). Toxicity was dependent on the relative level of expression of the HSV-1-tk gene, the concentration of nucleoside analogs to which cells were exposed, and cell division. The lymphoid system is well suited to test whether this technique can be used *in vivo* since many lymphocytes are replicating during differentiation and would, therefore, be susceptible to thymidine kinase ablation. In addition, a variety of cell surface markers have been identified that can be used in the analysis of cell lineage and function.

We have created transgenic mice harboring a construction consisting of the mouse immunoglobulin μ heavy chain enhancer and the κ light chain promoter fused to the HSV-1-tk gene (Fig. 1). This hybrid promoter/enhancer combination has been shown to be active when transfected into a mouse myeloma cell line (10). Twelve founder lines containing this transgene (referred to as KHTK) were generated. To verify that expression in these lines was limited to a restricted set of tissues, HSV-1-tk enzyme activity was assayed in tissue extracts from transgenic mice and their nontransgenic litter mates. Table 1 summarizes HSV-1-tk activity in various tissues from the founder lines, 552 and 563. Enzyme activity was present in spleen, thymus, bone marrow, and mesenteric lymph nodes of both lines. With the exception of the cerebral cortex in founder line 563, no enzyme activity was observed in other tissues assayed. There was no detectable enzyme activity in nontransgenic litter mates. This pattern demon-

FIG. 1. Diagram of immunoglobulin (Ig)-HSV-1-tk (KHTK) construct.

Table 1. Tissue distribution of HSV-1-tk enzyme activity in KHTK mice

Tissue	HSV-1-tk activity, pmol per min per mg of protein	
	Line 552	Line 563
Spleen	1.1	2.9
Thymus	5.5	5.7
Bone marrow	1.0	0.6
Lymph node	1.6	1.0
Cerebral cortex	<0.03	0.4
Liver	<0.03	<0.03
Kidney	<0.03	<0.03
Ovaries	<0.03	<0.03
Heart	<0.03	<0.03

Tissue extracts from KHTK mice were prepared and assayed for HSV-1-tk activity. Data are means of three determinations.

strates that HSV-1-tk expression is relatively lymphoid-specific, although not necessarily restricted to B-cell lineages.

The Pattern of Cell Ablation in Tissues Expressing HSV-1-tk. Transgenic mice from founder line 563 and their nontransgenic litter mates were treated with the nucleoside analog GCV for 1 week by using a miniosmotic pump. This delivery achieves a steady-state serum concentration of 1–5 μ M within 24 hr (as determined by HPLC). The concentration of GCV in all tissues assayed was comparable to this value. A separate control group was implanted with a miniosmotic pump containing isotonic phosphate-buffered saline (PBS). After 7 days of drug treatment, macroscopic examination of transgenic mice treated with GCV ($n = 22$) revealed a severe atrophy of the thymus, spleen, and mesenteric lymph nodes. The wet weights of the spleen and thymus were reduced to 15% of control and wet weights of lymph nodes were reduced to 20% of control. All nonlymphoid tissues were normal. Analysis of nontransgenic mice treated with GCV ($n = 16$) revealed no apparent pathology and wet weights of the internal organs were normal.

Consistent with the decreased organ weight, the number of cells in the spleen, bone marrow, and thymus of GCV-treated transgenic mice decreased to 15%, 15%, and 2% of control values, respectively (Fig. 2). In contrast, cell counts of lymphoid tissues of nontransgenic mice treated with GCV remained unchanged from the PBS-treated group. GCV treatment, therefore, selectively destroys tissues expressing HSV-1-tk with no apparent effect on tissues that do not express this enzyme.

The pathologic effect of GCV treatment was further evaluated by histological analysis. Serial cross sections through the thymus from PBS-treated mice reveal a normal thymic structure (Fig. 3A) that consists of a series of encapsulated lobules composed of a dark staining peripheral zone, the cortex, and a lighter staining central zone, the medulla. The thymus from nontransgenic mice treated with drug for 7 days appears normal (Fig. 3B). In contrast, GCV treatment of transgenic mice produced an atrophied thymus with a virtual complete absence of the cortex and a hypocellular medulla (Fig. 3C). This indicates that regions of the thymus that are known to contain the highest density of rapidly dividing thymocytes have been ablated. Additional histological analysis of spleen and mesenteric lymph nodes of transgenic and nontransgenic littermates treated with GCV revealed extensive cellular destruction only in transgenic mice.

Although the pattern of toxicity was clearly lymphoid-specific, it was not possible to evaluate the impact of GCV treatment on subpopulations of B and T cells by visual inspection. Flow cytometry was employed to analyze specific populations of B and T lymphocytes in mice subjected

FIG. 2. Effect of GCV treatment on cell number in lymphoid tissues of KHTK mice. Cell number in spleen (A), bone marrow (B), and thymus (C) are shown. Transgenic and nontransgenic mice from founder line 563 were treated with GCV for 7 days. The control group consisted of transgenic mice receiving PBS. Cell numbers have been normalized to the control group in which total number of cells is 9×10^7 in spleen, 3×10^7 in bone marrow, and 10^8 in thymus. These data are the means of six experiments. Statistical analysis reveals a significant difference ($P < 0.05$) between transgenic mice treated with GCV and either transgenic mice treated with PBS or nontransgenic mice treated with GCV. No statistical difference ($P > 0.05$) was observed between transgenic mice treated with PBS and nontransgenic mice treated with GCV. Bars: 1 (open bars), transgenic mice treated with PBS; 2 (hatched bars), nontransgenic mice treated with GCV; 3 (solid bars), transgenic mice treated with GCV.

to various GCV treatment protocols. B lymphocytes were identified by analyzing for the markers, immunoglobulin and B220, and T lymphocytes were identified by Thy-1. After 7 days of GCV treatment, striking and different effects were observed on B and T lymphocytes in spleens of KHTK mice (Fig. 4). The most dramatic impact was on immunoglobulin- and B220-positive B cells, which were reduced by a factor of 20 after GCV treatment. Cells bearing the T-cell surface marker, Thy-1, had decreased to 35% of the PBS group. Thus, although both B and T cells in the periphery were sensitive to the toxic action of GCV, B-cell lineages were more susceptible to ablation. Transgenic mice treated with PBS and nontransgenic mice treated with GCV had a normal profile of antigenic markers, indicating that neither HSV-1-tk expression by itself nor GCV treatment alone was sufficient to achieve toxicity.

A similar analysis was used to examine the impact of GCV treatment on thymocytes expressing the T-cell surface antigens L3T4 and Lyt-2 (Table 2). Mice were treated with GCV for 5 days and then two-color flow cytometry was performed to assess the effect of GCV treatment on double-positive, single-positive, and double-negative populations. In nontransgenic mice, 80% of cells were double-positive, 14% were single-positive, and 5% were double-negative. The composition of cells surviving GCV treatment in transgenic mice was markedly altered from that of the control population. There was a relative depletion by a factor of 4 in the double-positive L3T4⁺,Lyt-2⁺ population and a 3- to 5-fold increase in single-positive L3T4⁺,Lyt-2⁻ and L3T4⁻,Lyt-2⁺ cells. There was also an increase in the percentage of cells expressing T-cell receptor complex-associated molecule CD3 ϵ . These data imply a relative enrichment for more

FIG. 3. Histological analysis of the thymus of KHTK mice treated with GCV. Histological cross section through the thymus of a KHTK transgenic mouse treated with PBS (A), nontransgenic KHTK littermate treated with GCV (B), KHTK transgenic mouse treated with GCV (C). ($\times 15$; hematoxylin and eosin.) Mice from founder line 563 were treated for 7 days. M, medulla; C, cortex.

mature cells typical of populations in the thymic medulla (14, 15) and are in agreement with the histological data (Fig. 3). There was also a 6-fold increase in L3T4⁻,Lyt-2⁻ cells. Based on the distribution of other markers (data not shown), the L3T4⁻,Lyt-2⁻ population in the thymus of drug-treated mice is heterogeneous and includes cells with phenotypes that have been associated with early thymocyte progenitors (12, 16) as well as heat-stable antigen-negative CD3⁺ cells (17, 18), which do not have demonstrable progenitor activity (19). Two-color flow analysis (data not shown) indicates that L3T4⁻,Lyt-2⁻ cells comprise both phenotypic classes. It should be pointed out that, although single-positive and CD3⁺ cells are relatively enriched in the thymus of drug-treated animals (i.e., percentage), in terms of absolute cell number all thymic lineages are ablated in drug-treated transgenic animals.

Recovery of the Lymphoid System following GCV Treatment. One potential advantage of the conditional ablation method is the ability to remove the drug and evaluate the regenerative capabilities of the affected cell lineages. Repopulation of specific thymocyte lineages in KHTK mice was examined by subjecting mice to a severe ablation of lymphoid tissues followed by a recovery period. Accordingly, one group of animals was treated with GCV or PBS for 7 days, while a second group was treated with GCV or PBS for 7 days followed by a 7-day recovery period. All groups were assayed simultaneously for the total number of thymocytes and the presence of specific antigenic markers. Transgenic mice

FIG. 4. Ablation of B- and T-cell lineages in the spleen of KHTK mice treated with GCV. (A) Number of immunoglobulin (Ig)-positive cells. (B) Number of B220-positive cells. (C) Number of Thy-1-positive cells. Mice from the founder line 563 were treated with GCV for 7 days. Analysis of cells that express the antigenic markers Ig, B220, and Thy-1 was performed by flow cytometry. The number of cells expressing a given antigen was determined from the total cell count (Fig. 2) and percentage of antigen-positive cells determined by flow cytometry. In control mice (i.e., transgenic mice treated with PBS), 25% of the cells express Ig, 35% express B220, and 35% express Thy-1. These data are the means of three experiments. There was no difference between transgenic and nontransgenic mice treated with PBS (data not shown). Bars are as in Fig. 2.

treated with PBS had 1.1×10^8 thymocytes, whereas, after 7 days of GCV treatment, >99% of the thymocyte population was depleted. After 7 days of recovery, the total number of cells increased by 60-fold to nearly 50% of the PBS-treated control group. To characterize specific populations of thymocytes in the depleted and recovering thymus, one-color flow cytometry was performed (Fig. 5). GCV treatment of transgenic mice resulted in a large decrease in L3T4⁺ and Lyt-2⁺ cells. However, after a 7-day recovery, both L3T4⁺ and Lyt-2⁺ populations were very similar to control values, indicating that sufficient residual stem cells remained in the thymus and/or bone marrow to repopulate these lineages. Nontransgenic mice treated with GCV had the same percentage of L3T4⁺ and Lyt-2⁺ thymocytes as PBS-treated animals. The phenotype of the recovering nontransgenic animals

Table 2. Phenotype of T-cell lineages of KHTK mice after GCV treatment

Markers	% thymocytes	
	Nontransgenic	Transgenic
L3T4 ⁺ ,Lyt-2 ⁺	80	21
L3T4 ⁺ ,Lyt-2 ⁻	10	27
L3T4 ⁻ ,Lyt-2 ⁺	4	21
L3T4 ⁻ ,Lyt-2 ⁻	5	30
CD3-ε ⁺	35	76
Total cell number	6.3×10^7	3.8×10^6

Mice of transgenic line 552 or nontransgenic littermates were treated with GCV for 5 days. The thymuses of individual mice were assayed by two-color flow cytometry and reported as a percentage of total thymocytes expressing the indicated markers. Data are typical of three experiments.

FIG. 5. Recovery of thymic populations in KHTK mice after ablation. (A) Percent of L3T4-positive thymocytes. (B) Percent of Lyt-2-positive thymocytes. Mice from founder line 552 were treated for 7 days. Analysis of thymocytes that express L3T4 and Lyt-2 was performed by flow cytometry. Bars: 1, transgenic mice treated with PBS; 2, transgenic mice treated with GCV; 3, transgenic mice treated with GCV and allowed to recover for 7 days after drug treatment; 4, nontransgenic mice treated with GCV; 5, nontransgenic mice treated with GCV and allowed to recover for 7 days after drug treatment.

was the same as the control group, indicating that GCV treatment had no nonspecific effects on T-cell lineages.

DISCUSSION

These results describe the development of a toxic vector system that exploits transgenic animal technology to achieve conditional ablation of targeted cell types. This system depends on introduction of HSV-1-tk into the mouse germ line under control of cell-specific promoters. The toxicity results from metabolism of specific nucleoside analogs to toxic intermediates by HSV-1-tk. This approach is dependent on two experimental parameters. (i) The spatially restricted tissue expression of HSV-1-tk which can be controlled by choosing an appropriate tissue-specific promoter, and (ii) the temporally restricted administration of the nucleoside analog. This latter condition allows control of both the precise time of destruction by choosing when to deliver the drug and the degree of cell ablation by varying the dose or duration of drug treatment. A final consideration is that ablation is directly proportional to the rate at which cells are undergoing cellular division.

We chose to target HSV-1-tk expression to the lymphoid system of transgenic mice to examine the specificity of this conditional ablation approach *in vivo*. The KHTK transgenic mice expressed HSV-1-tk enzyme in lymphoid tissues of B- and T-cell lineages. This lymphoid specificity may reflect a common regulatory component for genes unique to B- and T-cell lineages. A similar pattern of expression has been observed in transgenic mice using a similar hybrid immunoglobulin promoter/enhancer (20). From the patterns of enzyme activity observed in the KHTK mice, one would have predicted that the spleen, thymus, bone marrow, and lymph nodes would be susceptible to the cytotoxic action of GCV. This prediction has been borne out in all lymphoid tissues examined, as determined by cell number, histological analysis, and flow cytometry.

In KHTK mice, cells of B- and T-cell lineages are readily eliminated by GCV treatment. This depletion is readily apparent after 2 days of GCV treatment. Splenic B cells show relatively more complete ablation than those of the T-cell lineage. This may be due to a quantitative difference in either the level of HSV-1-tk expression in B and T cells and/or in the

rate of cell division of the two lineages. A difference in turnover rate of B and T lymphocytes is supported by studies of Freitas *et al.* (21), demonstrating that $\approx 90\%$ of cells in peripheral B compartments are turning over relatively rapidly and the remaining 10% of B lymphocytes compromise a long-lived compartment indicative of a low turnover rate. In contrast, only $\approx 50\%$ of peripheral T lymphocytes show a rapid turnover rate (21). The high degree of ablation observed in bone marrow suggests that some hematopoietic cells in addition to T and B cells may also be sensitive to ablation.

Histological analysis and flow cytometry indicate that although all thymic lineages are depleted, there is a relatively more complete depletion of double-positive ($L3T4^+$, $Lyt-2^+$) cells, typical of the thymic cortex. This result is consistent with data showing that the most rapidly proliferating cells are located in the cortex (22, 23) and would therefore be most sensitive to thymidine kinase ablation. A relative sparing of mature single-positive cells, located primarily within the medulla, is consistent with a lower mitotic rate within this region of the thymus (22).

$L3T4^-$, $Lyt-2^-$ cells are also relatively enriched in the thymus of drug-treated animals. It is known that this population contains thymocyte progenitors capable of repopulating the thymus of an irradiated animal (24). We demonstrate that the thymus of a drug-treated animal is repopulated within 7 days after drug treatment. The $L3T4^-$, $Lyt-2^-$ population is known to contain many cells that are not in cycle (23, 25), and it is reasonable to assume that some nondividing progenitor cells are spared and are capable of repopulating the thymus within 7 days. We cannot exclude the possibility that some progenitors may be spared because they do not express the HSV-1-tk gene or that progenitors in the bone marrow may have contributed to thymic repopulation. It is clear from the recovery studies, however, that it should be possible to devise conditions that will allow the study of detailed events that occur during repopulation from a limited number of residual cells. Should specific populations of stem cells be spared after ablation, this approach may be useful to enrich for and isolate them.

An alternative cell-ablation approach in transgenic mice has been demonstrated by expressing the diphtheria toxin A chain or ricin A chain under control of tissue-specific promoters (26–29). Expression of the diphtheria toxin or ricin gene resulted in destruction of specific cell types in pancreas, lens, or pituitary. Although extremely toxic and not dependent upon cell division, the utility of this approach may be compromised due to the difficulty in generating transgenic pedigrees and the inability to control the time at which ablation occurs. The constitutive nature of the toxin-gene expression may result in the embryonic ablation of organs that would produce a lethal phenotype (26) and may account for the apparent low penetrance of the toxic phenotype (26, 28, 29).

The advantages of thymidine kinase ablation include its ability to control the time and degree of cell ablation. By controlling these parameters, it should be possible to study cell lineage relationships and determine the precise time at which cells become committed to a particular developmental fate. In addition, the ability to stop the drug-induced toxicity allows the evaluation of stem cell and organ plasticity. Finally, since expression of HSV-1-tk is nontoxic, it allows transgenic pedigrees to be easily established. The thymidine kinase obliteration approach should, therefore, be amenable to a variety of biological systems in which cells are undergoing division and allow for the creation of transgenic hypomorphs whose phenotypes result from the ablation of specific cell types.

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