Animal Model

An HSV-TK Transgenic Mouse Model to Evaluate Elimination of Fibroblasts for Fibrosis Therapy

Bin Tian, Lei Han, Jill Kleidon, and Craig Henke

From the Department of Medicine, University of Minnesota,
Minneapolis, Minnesota

Pathological fibroproliferation after tissue injury is harmful and may lead to organ dysfunction. Unfortunately, fibroproliferative diseases remain intractable to current therapeutic strategies. Thus, new therapeutic approaches are needed. One possible approach is to promote resolution of physiological fibroproliferation that follows injury before it becomes pathological by activating apoptosis selectively in fibrotic lesions. However, it is not known whether selective elimination of fibroblasts will prevent fibrosis or impede repair or worsen injury by eliminating topographic signals essential to organ reconstitution. To address this question, a tractable *in vivo* model system is needed in which fibroblasts can be targeted to undergo apoptosis at a chosen time and place. We developed transgenic mice expressing HSV-TK from the type I collagen promoter to determine whether selective elimination of fibroblasts actively forming fibrotic lesions is an effective therapeutic strategy for fibroproliferative disorders. The transgene renders fibroblasts actively forming fibrotic tissue susceptible to ganciclovir. To validate the transgenic model we examined whether administration of ganciclovir prevents the development of fibrosis in sponges implanted subcutaneously in the backs of the transgenic mice. We demonstrate that fibroblasts/myofibroblasts isolated from sponges express HSV-TK protein and are selectively ablated by ganciclovir *in vitro*. In adult transgenic mice, ganciclovir treatment attenuated the development of fibrotic tissue in the sponges both biochemically and histologically. We conclude that this transgenic model system is an ideal approach to determine whether targeted ablation of fibroblasts is an effective therapeutic strategy for fibrotic diseases. (*Am J Pathol* 2003, 163:789–801)

Fibroproliferative diseases can cause dysfunction of every major organ system and as a class of disorders is a major cause of death in the United States.¹ Examples of

fibroproliferative disease states include pulmonary fibrosis, cirrhosis, cardiac fibrosis, luminal occlusion after coronary artery angioplasty, glomerulosclerosis, and renal fibrosis. Each of these fibroproliferative disorders is characterized by injury resulting in alterations in normal parenchymal cell and connective tissue architecture by fibroblasts/myofibroblasts and their connective tissue products causing progressive organ dysfunction and, frequently, death.^{2–8} As a class of disorders, fibroproliferative diseases remain intractable to current therapeutic options.⁹ Based on advances in contemporary fibroblast biology novel therapeutic strategies for interdicting the fibroproliferative disorders can now be examined. One potential approach focuses on promoting the resolution of physiological fibroproliferation that follows tissue injury before it results in permanent fibrosis. This may be possible to accomplish by activating apoptosis selectively in newly formed fibrotic lesions.

Fibroblasts/myofibroblasts are the central effector cell resulting in organ fibrosis.^{7,8,10–13} Evidence indicates that myofibroblasts are activated fibroblasts and synthesize high levels of collagen.^{10,14} They constitute the fibroblastic foci of developing fibrotic lesions.^{8,14} A large body of data supports the concept that, during normal organ repair, resolution of fibroproliferation takes place when the physiological mechanism for elimination of fibroblasts/myofibroblasts, apoptosis, occurs in a timely fashion.^{11,15–22} However, no studies have been performed to examine whether the selective ablation of the myofibroblasts during the physiological fibroproliferation which results after injury will attenuate the development of organ fibrosis, impede repair, or worsen injury. To investigate whether the selective elimination of myofibroblasts actively forming fibrotic lesions is an effective therapeutic strategy for fibroproliferative diseases, a tractable *in vivo* model is needed in which myofibroblasts can be killed at

Supported by the Minnesota Medical Foundation, an American Heart Association Grant-In-Aid, an American Lung Association Career Investigator Award, and National Institutes of Health grants RO1 HL67794 and P50HL50152.

Accepted for publication May 5, 2003.

Address reprint requests to Craig A. Henke, M.D., Box 276, University of Minnesota, 420 Delaware Street SE, Minneapolis, MN 55455. E-mail: henke002@umn.edu.

a precise time and location. To address this issue, we have developed transgenic mice expressing HSV-TK from the type I collagen promoter. Cells actively producing HSV-TK metabolize the antiviral agent ganciclovir (GCV) to toxic nucleotide analogs that promote cell death.^{23,24} A property of fibroproliferative fibroblasts/myofibroblasts is active type I collagen production.^{8,25–29} Thus the transgene renders fibroblasts actively forming fibrotic lesions sensitive to GCV permitting us to therapeutically trigger fibroblast/myofibroblast apoptosis in evolving fibroblastic foci. Here we report characterization of the transgenic mice and validation of the model system. To validate this transgenic mouse model we have examined whether administration of GCV prevents the development of fibrotic tissue in sponges implanted subcutaneously into the backs of the transgenic mice. We demonstrate that *in vitro* sponge/wound fibroblasts/myofibroblasts are selectively ablated by GCV and that *in vivo* transgenic mice treated with GCV both biochemically and histologically have reduced fibrotic tissue within the sponge material compared to mice treated with saline. Our data indicate that this model system is an ideal approach to determine whether ablation of fibroblasts/myofibroblasts is an effective therapeutic strategy for acute or chronic fibrotic disease.

Materials and Methods

Type I Collagen-HSV-TK/GFP Fusion Gene Construct

The EGFP fragment in the promoter-less vector pEGFP-1 was replaced by the fragment IRES-EGFP from the vector pIRES2-EGFP (Clontech, Palo Alto, CA). Next, the 1.5-kb HSV-TK cDNA coding sequence was amplified by primer chain reaction (PCR) from the plasmid pTK-1 (gift from Dr. Victor Canfield, Penn State College of Medicine). The 5' primer (GGATCTTGGTCTGACTGAACTCCCG) is located at –58 bp and generated a *Sal*I site. The 3' primer (GTCATAGCGCGGATCCTTCCGG) is located at +1172 bp right before the transcriptional termination signal and generated a *Bam*HI restriction site. The PCR-generated TK fragment was subcloned into the *Sal*I-*Bam*HI site of the multiple cloning site of vector pIRES-EGFP. A 6-kb fragment of the mouse α 2 type I collagen enhancer (*Bgl*II-*Bgl*II, –19.3 to –13.5 kb) and the “minimal” α 2 type I collagen promoter (–350 to +54 bp) (plasmid pRM350–6kb-lacZminus, a gift from Dr. Benoit de Crombrughe, M.D. Anderson Cancer Center) was placed in front of the TK sequence by blunt-end ligation to generate the expression cassette plasmid pCol1-tk/IRES-EGFP (Figure 1A). The col1-HSV-TK/GFP fusion gene construct was excised and purified by gel electrophoresis.

Animals

The C57BL/6 strain of mice, which is bleomycin-sensitive, was used for microinjections. Microinjections were performed by Dr. Thomas Wagner (Oncology Research In-

Figure 1. *In vivo* expression of the type I collagen-HSV-TK transgene. **A:** α 2 type I collagen-HSV-TK/GFP fusion gene construct. It is a bicistronic construct consisting of the HSV-TK cDNA separated from EGFP cDNA by IRES. The construct is under the control of the α 2 type I collagen far upstream enhancer linked to the minimal α 2 type I collagen promoter. **B:** Southern blot of *Bam*HI-digested genomic DNA from founder mice and non-transgenic littermates. P and N denote positive and negative controls, respectively. **C:** Western blot of whole lung extracts from transgenic progeny of founder line 21 following bleomycin-induced lung fibrosis. Bleomycin (2 units/kg) was instilled intratracheally and the lungs were harvested 3 weeks later for examination of HSV-TK protein expression. HSV-TK migrates at 45 kD with several minor bands at ~40 to 43 kD as has been previously described.³¹

stitute, Greenville, South Carolina). DNA obtained by tail biopsy from resulting mice were digested with *Bam*HI and screened by Southern blot using the 1.5-kb HSV-TK probe. Six- to eight-week-old adult animals were used for all experiments.

Surgical Procedures and Tissue/Sponge Harvest

Surgical procedures were performed under anesthesia with ketamine (8 mg/100 g; Ketamine, Phoenix Pharmaceutical, Inc., St. Joseph, MO) and xylazine (2 mg/100 g). Sponges were subcutaneously implanted into the backs of the transgenic mice as previously described.⁵ To harvest sponges or organ tissue, the mice were killed by terminal barbiturate anesthesia. The sponge/organ tissues were either frozen in liquid nitrogen and stored at –70°C or fixed in 4% paraformaldehyde and processed for hematoxylin and eosin (H&E)- or trichrome-stained paraffin sections.

GCV Administration

GCV diluted in sterile saline was administered continuously at a rate of 100 mg/kg/day for 14 days via subcutaneously implanted mini-osmotic pumps (Alzet, Model 2002). This dose has been reported to kill cells expressing HSV-TK in transgenic mice^{30,31}

Sponge Cell Isolation and Characterization

Sponge/wound cell cultures were developed by mechanical and enzymatic dispersion of sponge/wound tissue as previously described.^{5,32} The cells were cultured in DMEM + 10% FCS. Subcultivation was performed weekly at a split ratio of 1:3. Cells were used for studies at passages 2 and 3. The sponge/wound cells grown on tissue culture plastic were characterized by morphological and immunological criteria. Primary antibodies used to characterize the sponge cells/tissue included anti- α -smooth muscle actin (Sigma Chemical Co., St. Louis, MO), anti-vimentin (Sigma), anti-CD36 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-Factor VIII (Accurate Chem. & Sci. Corp., Westbury, NY), and anti-HSV-TK (a gift from Dr. William Summers, Yale University).

Western Analysis

Sponge/wound tissues were powdered in liquid nitrogen, lysed with lysis buffer containing protease inhibitors, and centrifuged at $10,000 \times g$. Equal amounts of protein from the cell lysates (supernatant proteins) were separated on SDS-PAGE and transferred to nitrocellulose membranes as described.³³ The primary antibody for HSV-TK expression was a polyclonal rabbit anti-HSV-TK antibody obtained from Dr. William Summers, Yale University.

Histology and Immunohistochemical Analysis

Sponge/wound tissues were fixed with 4% paraformaldehyde and paraffin sections stained by H&E or trichrome. Immunohistochemical analysis was performed as previously described.^{34,35} Briefly, for immunofluorescent staining paraffin-embedded tissue sections were dewaxed, rehydrated, and permeated (1X tris-buffered saline (TBS) containing 0.5% Triton X-100 for 5 minutes) before blotting with blotting buffer (1X TBS containing 2% bovine serum albumin (BSA) and 0.25% Tween 20 for 1 hour at 37°C). The procedures for cultured cells were the same as that for tissue except that the cells were fixed directly with 100% cold methanol for 10 minutes. The slides were then incubated with appropriate primary antibodies in blotting buffer. The slides were washed three times (1X tween tris-buffered saline (TTBS), 15 minutes each wash at 37°C), incubated with the appropriate fluorescent-labeled secondary antibodies (1:200 dilution) or Hoescht stain in blotting buffer (1 hour at 37°C) followed by three additional washes. The image data were collected using fluorescent phase-contrast and confocal microscopic systems. For immunohistochemical staining the procedures were the same as described above

except that horseradish peroxidase (HRP)-labeled secondary antibodies were used. After incubation with HRP-conjugated secondary antibodies the signal was visualized by incubating the slides with 3-3'-diaminobenzidine (DAB; Sigma).

Hydroxyproline Assay

Hydroxyproline content was assessed as previously reported.^{36,37} Frozen sponge/wound tissues were powdered and digested with 6 N HCL at 110°C for 16 hours. The supernatant was neutralized with equal volume of 6 N NaOH and dried at 70°C in an incubator. The samples were resuspended in 0.2 ml of water followed by addition of equal volume of 0.05 mol/L chloramine-T and incubated at room temperature for 25 minutes. Then 0.2 ml of 3.15 mol/L perchloric acid and Erlich's reagent were added. The samples were mixed well and incubated at 60°C in water bath for 20 minutes and read in a plate reader at 560 nm, and the amount of hydroxyproline was calculated from a standard curve constructed using hydroxy-L-proline at concentrations of 0.25 to 40 μ g/ml. The final hydroxyproline content was adjusted according to the wet weight of the sponge tissue.

Statistical Analysis

All data are expressed as means $\pm$ SE. Experiments were performed three times. Paired evaluations were made for experimental and control conditions within each experiment and significance was determined by Student's *t*-test. Significance level was set at $P < 0.05$.

Results

Generation of HSV-TK-Expressing Transgenic Mice

Progeny resulting from the pronuclear injection of the α 2 type I collagen enhancer/promoter-HSV-TK/GFP fusion gene construct (coll-HSV-TK/GFP) were screened by Southern analysis for successful integration of the transgene (Figure 1B). Transgenic lines were established from four founder mice. To identify which line of animals expressed the highest level of HSV-TK protein during a fibroproliferative response, bleomycin (2 units/kg) was instilled intratracheally, and after 3 weeks the lungs were harvested and whole lung extracts were examined by Western analysis. Of these, line 21 displayed the highest and most consistent expression of HSV-TK protein during the fibroproliferative response after bleomycin-induced lung fibrosis (Figure 1C). Males of line 21 had fertility problems in accord with prior reports of male infertility in HSV-TK-expressing transgenic mice.³⁸ Experimental mice were generated by mating heterozygous females from line 21 with wild-type males, followed by genotyping by Southern blot and demonstration of HSV-TK expression by Western analysis. Therefore, the genetic back-

Figure 2. *In vitro* characterization of transgenic sponge/wound cells. Sponges were implanted subcutaneously into the backs of the transgenic mice and were harvested 21 days later. Cell cultures were prepared as described in the Materials and Methods section. Shown are the confocal microscopic appearance of immunostained cells isolated from sponges 21 days after implantation. **A:** Double immunofluorescence analysis of day 21 isolated untreated transgenic sponge cells (**panels A–C**) and GCV-treated cells (**panels D and E**) (magnification, $\times 200$). **Panel A:** Anti- α -smooth muscle actin (green) and anti-vimentin (red). **Panel B:** Anti- α -smooth muscle actin (green) and anti-CD36 (red). The α -smooth muscle actin-positive cells (green) stained negative for CD36 (red). **Panel C:** Anti-vimentin (red) and anti-CD36 (green). Cells staining positive for both appear yellow (**arrowheads**). **Panel D:** Anti- α -smooth muscle actin (green) and anti-vimentin (red). **Panel E:** Anti-vimentin (red) and anti-CD36 (green). Cells staining for both appear yellow (**arrowheads**). **Panel F:** DAB immunostaining for HSV-TK (magnification, $\times 600$; **arrowhead**). Shown in the **inset** is colocalization of HSV-TK expression with α -smooth muscle actin (green). **B:** Immunostaining analysis of isolated untreated transgenic sponge cells (**panels A and B**) and cells treated with GCV for 5 days (**panels C and D**) using anti-vimentin (**panels A and C**, green) and anti-HSV-TK (**panels B and D**, red). **Panels A–D** represent identical cells stained with either anti-vimentin or anti-HSV-TK. Note in **panel C** that the remaining cells were vimentin-positive but were negative for HSV-TK (**panel D**). **Panel E** is HSV-TK immunostaining of nontransgenic sponge cells as a control. Some background staining is apparent.

Figure 3. Vulnerability of transgenic sponge fibroblasts/myofibroblasts to GCV *in vitro*. **A:** HSV-TK Western analysis of whole sponge extracts from transgenic (T) and non-transgenic (NT) mice. Only sponge/wound cells derived from transgenic mice express HSV-TK protein. **B:** Phase contrast picture of transgenic sponge cells treated with various doses of GCV for 5 days (0, 0.02, 0.5, 0.2, 2 and 5 $\mu\text{mol/L}$; panels A-F, respectively) **C:** Transgenic and nontransgenic sponge fibroblasts/myofibroblasts were exposed to GCV in varying concentrations for 5 days. The number of viable cells were quantified by Hoescht staining (bis-benzimide, Hoescht 33258).

grounds of transgenic and nontransgenic control mice were similar.

Coll-HSV-TK Transgenic Sponge/Wound Myofibroblasts Are Susceptible to GCV Mediated Killing in Vitro

This study was designed to validate our HSV-TK transgenic mice as a model system to determine whether selective elimination of fibroblasts is a valid treatment approach for fibroproliferative disorders. We chose a sponge implantation approach to validate our model. The sponge approach was chosen because the sponges before implantation do not contain type I collagen, elim-

inating any background collagen that may make it difficult to assess the effect of GCV on developing fibrotic tissue. In addition, after subcutaneous implantation, the sponges become organized by fibrotic tissue after several weeks. The sponges are easy to remove thus permitting isolation and characterization of the cells involved in the sponge-induced fibrotic response. Furthermore, both hydroxyproline analysis and trichrome staining can be performed on the sponge/wound tissue. Therefore, the sponge approach is ideal for validating our transgenic mice as a tool to assess the efficacy of ablation of fibroblasts as anti-fibrotic therapy.

Cells were isolated from sponges 21 days after sponge implantation. Isolated cells were characterized morphologically by phase contrast and confocal microscopy followed by immunostaining for cell identification and HSV-TK expression. Phase contrast and confocal microscopy demonstrated a mixed population of cells morphologically (Figure 2A, panel A). Many of the cells before GCV treatment were large, well-spread cells with the typical elongated, spindle shape of fibroblasts/myofibroblasts. There were also many smaller cells with a more round appearance. Immunohistochemical studies indicated that the majority of the large, well-spread cells isolated from sponges derived from transgenic mice expressed determinants characteristic of fibroblasts/myofibroblasts (α -smooth muscle actin and vimentin) but were negative for endothelial cell determinants (CD36) (Figure 2A, panels A and B). These large, well-spread α -smooth muscle actin-positive cells also stained positive for HSV-TK (Figure 2A, panel F). Immunostaining indicated that many of the smaller, more round cells had determinants characteristic of endothelial cells (CD36 and vimentin-positive and α -smooth muscle actin-negative) (Figure 2A, panels B and C). These cells did not stain positive for HSV-TK. Similar results were obtained using sponge cells isolated from nontransgenic control mice except that nontransgenic fibroblasts/myofibroblasts did not stain positive for HSV-TK (Figure 2B, panel E).

We examined our transgenic and nontransgenic wound/sponge cells for HSV-TK expression by Western analysis. Western blots demonstrated that only wound/sponge cells from transgenic mice expressed HSV-TK protein (Figure 3A). To examine the vulnerability of transgenic and nontransgenic sponge/wound cells to GCV, subconfluent, proliferating cultures of sponge/wound cells derived from HSV-TK transgenic mice were exposed to GCV at varying concentrations (0, 0.02, 0.2, 0.5, 2, and 5 $\mu\text{mol/L}$) for 5 days. Sponge/wound cells isolated from transgenic mice were killed by GCV in a dose-dependent fashion (Figure 3, B and C). Exposure to GCV (2 $\mu\text{mol/L}$ for 5 days) caused a mean cell loss of ~60% in cultures of transgenic sponge/wound cells (Figure 3C). The intensity of kill was such that after 5 days of GCV there was an ~40% reduction in transgenic sponge/wound cell number with the lowest concentration of GCV tested (0.02 $\mu\text{mol/L}$). In contrast, sponge/wound cells isolated from nontransgenic littermate mice and exposed to GCV remained largely viable (Figure 3C). However, there was evidence of non-specific toxicity of GCV to

Figure 4. Histological analysis of sponge material from transgenic animals treated with either saline or GCV for 14 days (days 7 through 21 after sponge implantation). Sponges were harvested on day 21. **A** and **B**: H&E staining of sponge tissue from a transgenic mouse after treatment with saline. Magnification, $\times 100$ and $\times 400$, respectively. **C** and **D**: H&E staining of sponge tissue from a transgenic mouse after treatment with GCV. Magnification, $\times 100$ and $\times 400$, respectively. **E** and **F**: H&E staining of sponge tissue derived from a nontransgenic mouse after 14 days of GCV. Magnification, $\times 100$ and $\times 400$, respectively.

nontransgenic cells especially with the highest dose of GCV tested ($5 \mu\text{mol/L}$).

We also characterized the transgenic sponge cells that survived 5 days of GCV treatment to identify which cells were susceptible to GCV. Morphologically, the cells remaining after GCV also consisted of a mixed population. However, in marked contrast to pre-GCV treatment in

which many of the cells were large, well-spread, and elongated (Figure 2A, panel A), after GCV treatment the majority of remaining cells were smaller and less spread in appearance (Figure 2A, panel D). The vast majority of the larger well-spread cells with determinants characteristic of fibroblasts/myofibroblasts (α -smooth muscle actin- and vimentin-positive) had been eliminated (Figure

Figure 5. *In vivo* immunohistochemical characterization of sponge/wound fibrotic tissue. Immunofluorescence staining (magnification, $\times 200$) of sponge tissue from saline (A–C) and GCV (D–F) treated mice with anti- α -smooth muscle actin (A and D, green), anti-vimentin (B and E, red) and combined (C and F).

2A, compare panels A and D). Moreover, virtually all HSV-TK-expressing cells were eliminated (Figure 2B, compare panels B and D). However, a population of vimentin-positive cells not expressing HSV-TK remained after GCV (Figure 2B, panel C). Further characterization of these cells by immunostaining indicated that many of the remaining smaller, round cells had determinants characteristic of endothelial cells (CD36- and vimentin-positive and α -smooth muscle actin-negative) (Figure 2A, panel E). There were also a few cells after GCV that were vimentin-positive and CD36-negative. It is unclear whether these cells were quiescent fibroblasts that did not express HSV-TK or myofibroblasts resistant to GCV. Collectively, the *in vitro* data indicate that GCV preferentially targets HSV-TK transgenic fibroblasts/myofibroblasts for kill but not nontransgenic fibroblasts/myofibroblasts and appears to spare endothelial cells.

Subcutaneous GCV Treatment Attenuates the Development of Fibrotic Tissue in Sponges Implanted in Coll-HSV-TK Transgenic Mice

The ability of continuously administered GCV to kill fibroblasts/myofibroblasts and limit the development of fibrotic tissue was examined by implanting sponges subcutaneously into the backs of the transgenic mice. Fibroblast/myofibroblast invasion into and organization of sponge tissue requires several weeks.⁵ In addition, our *in vitro* experiments indicated that prolonged exposure to GCV was required to achieve significant ablation of trans-

genic sponge/wound cells. Therefore, GCV (or saline for controls) was delivered continuously *in vivo* by subcutaneous osmotic minipump from days 7 through 21 after sponge implantation. At day 21 the sponges were removed. Histological and biochemical analysis of sponge/wound tissue was performed to determine the effect of GCV on fibrotic tissue development.

The development of fibrotic tissue in the sponge material was robust in transgenic mice treated with sterile saline (the vehicle for GCV). Shown in Figure 4A and B, H&E staining revealed the presence of plump islands of newly formed fibrotic tissue interspersed between sponge material. Immunostaining demonstrated that the cellular composition of the fibrotic tissue consisted of predominantly vimentin- and α -smooth muscle actin-positive cells, determinants characteristic of fibroblasts/myofibroblasts (Figure 5A–C). Importantly, immunohistochemical analysis revealed the presence of abundant α -smooth muscle actin- and HSV-TK-positive cells in the islands of fibrotic tissue in sponges treated with saline (Figure 6A, panels A–C). In contrast, H&E staining of sponge tissue derived from transgenic mice treated with GCV for 14 days demonstrated the presence of sparse, non-nucleated cells and scant, thin strands of mostly acellular fibrotic tissue (Figure 4, C and D). Furthermore, immunostaining showed a reduction in vimentin- and α -smooth muscle-positive cells (Figure 5, D–F) and the virtual disappearance of α -smooth muscle actin- and HSV-TK-positive cells following 14 days of GCV (Figure 6A, panels D–F) suggesting that fibroblasts/myofibro-

Figure 6. Immunohistochemical analysis of the effects of GCV on HSV-TK expression in sponges *in vivo*. **A:** Immunofluorescence studies (magnification, $\times 200$) of HSV-TK expression in transgenic mice treated with saline (panels **A–C**) and GCV (panels **D–F**) using anti-HSV-TK (**A** and **D**, red), anti- α -smooth muscle actin (**B** and **E**, green), and combined (**C** and **F**). Panels **G** and **H** are negative controls without primary antibodies. **B:** DAB immunostaining (magnification, $\times 400$) of sponge tissue to analyze HSV-TK expression in saline treated- (panel **A**) and GCV treated- (panel **B**) transgenic mice.

blasts were eliminated by GCV. Shown in Figure 6A, panels G and H are negative controls (no primary antibody). No significant immunoreactivity of transgenic sponge tissue was apparent in the negative controls. To confirm our confocal immunofluorescence studies, we also immunostained sponge tissue from saline- and GCV- treated animals using DAB to analyze the effect of GCV on HSV-TK expression. Consistent with our immunofluorescence data we found that GCV reduced the number of HSV-TK-positive cells in the sponge fibrotic tissue (Figure 6B).

As a semi-quantitative measure of the effects of GCV on HSV-TK expression, whole sponge extracts derived from transgenic mice receiving either saline or GCV were analyzed by Western blot. Western analysis demonstrated that HSV-TK protein expression in sponge/wound tissue was not detectable in GCV-treated transgenic mice compared to saline-treated transgenic mice (Figure 7). Western analysis was also performed to semi-quantitatively measure the effect of GCV on α -smooth muscle actin expression and therefore serving as a rough mea-

Figure 7. Effects of GCV on HSV-TK and α -smooth muscle actin expression. Western analysis of whole sponge extracts from saline- and GCV-treated transgenic mice using a polyclonal HSV-TK antibody and a monoclonal α -smooth muscle actin antibody. Shown are the results from two separate sponge experiments. HSV-TK protein immunoreactivity is at 45 kD. α -smooth muscle actin immunoreactivity is at 43 kD.

sure of the effect of GCV on fibroblast/myofibroblast numbers. Western analysis of whole sponge extracts showed that α -smooth muscle actin expression markedly decreased after GCV treatment compared to saline-treated mice (Figure 7). Our *in vivo* data are consistent with the *in vitro* results and strongly suggest that fibroblasts/myofibroblasts actively involved in the fibroproliferative response are preferentially eliminated by GCV.

As a control, GCV was administered for 14 days to nontransgenic littermate mice in whom sponges had been implanted. In contrast to transgenic mice, abundant fibrotic tissue was present in the sponge material indicat-

ing that GCV had no detectable effect on the formation of fibrotic tissue in sponges implanted in nontransgenic littermates (Figure 4, E and F, H&E stain; Figure 8A, panels E and F, trichrome stain). Immunostaining analysis of sponge tissue from nontransgenic mice showed a similar distribution of α -smooth muscle actin- and vimentin-positive cells in the newly formed fibrotic tissue (data not shown). Immunostaining with anti-HSV-TK showed background staining only.

Histological analysis by trichrome staining of sponges obtained from transgenic mice treated with saline showed marked collagen deposition within the islands of fibrotic tissue interspersed between the sponge material at day 21 (Figure 8A, panels A and B). In contrast, in transgenic mice treated with GCV for 14 days there were scant, thin strands of collagen fibrils deposited in between the sponge material (Figure 8A, panels C and D). To confirm the trichrome staining findings and to quantitatively measure the amount of collagen deposited in the sponge/wound tissue, hydroxyproline analysis was performed. The hydroxyproline content of sponges derived from transgenic mice treated with saline for 14 days was $1634.6 \pm 380.8 \mu\text{g/g}$ of sponge tissue. The hydroxyproline content of sponges obtained from transgenic mice treated with GCV for 2 weeks was decreased >3 -fold

Figure 8. Effects of GCV on sponge collagen content. **A:** Trichrome staining of sponge tissue from saline- (**panels A and B**) and GCV- (**panels C and D**) treated transgenic mice. Magnification, $\times 100$, **panels A and C** and $\times 100$, **panels B and D**, respectively. Trichrome staining of sponge tissue derived from a nontransgenic mouse treated with GCV for 14 days (**panels E and F**). **B:** Hydroxyproline content of sponge tissue from saline- and GCV treated-transgenic mice. *, $P < 0.02$.

Figure 9. Western analysis of whole organ extracts (partial extract for skin) of major organs (heart, lung, aorta, liver, kidneys, GI tract, and skin) using a polyclonal HSV-TK antibody. **Upper panel:** Immature developing transgenic mice (4 weeks of age). **Lower panel:** Mature fully developed transgenic mice (4 months of age).

compared with saline-treated mice ($531.5 \pm 237.6 \mu\text{g/g}$ of sponge tissue; $P < 0.02$) (Figure 8B). Collectively, these data demonstrate that GCV administration to our HSV-TK transgenic mice kills HSV-TK-expressing fibroblasts/myofibroblasts *in vitro* and attenuates the development of fibrotic tissue in the implanted sponge material.

We did not examine the skin incision wound that was created to insert the sponges subcutaneously because removal of the sponges altered its integrity. However, we did not note any differences in wound closure between GCV- and saline-treated mice. It should be noted that the purpose of this study was not to examine in a comprehensive fashion the effect of fibroblast ablation on wound healing. Instead, the purpose was to validate the transgenic mouse model by examining whether GCV treatment can eliminate fibroblasts/myofibroblasts involved in a fibroproliferative response.

Administration of GCV for 14 Days Did Not Cause Overt Organ Abnormalities in Uninjured Transgenic Mice

Although the transgene should preferentially eliminate those fibroblasts actively forming fibrotic tissue (eg, actively producing type I collagen), non-specific toxicity to other organs was a concern given that HSV-TK expression was driven by the $\alpha 2$ type I collagen enhancer/promoter. Of specific concern was the skin and lung where collagen turnover is felt to be high compared to other organs.²⁸ To begin to address this issue, we performed Western analysis on whole organ extracts (partial extract for skin) of major organs (heart, aorta, lung, liver, kidney, GI tract, and skin) from uninjured immature, developing 4-week-old transgenic mice and mature, adult, 4-month-old transgenic mice to examine the level of HSV-TK expression. In developing mice, HSV-TK expression was present in all major organs with the skin, lung, and aorta showing the highest level of expression (Figure 9). In contrast, Western blots demonstrated that in fully developed mice only the lung and skin displayed some HSV-TK expression (Figure 9). However, HSV-TK protein expression in other major organs was either barely detectable or not detectable at all. No gross or histopathological abnormalities were detected in major organs including the skin and lung in untreated adult transgenic

mice or in uninjured adult transgenic mice treated with GCV for 14 days (data not shown). Furthermore, uninjured adult transgenic mice treated with GCV for 14 days appeared healthy and had stable body weight. Therefore, 2 weeks of GCV treatment did not cause overt, gross evidence of organ toxicity in uninjured adult transgenic mice although less readily identifiable organ injury remains a possibility.

It is important to note that following organ injury and induction of a fibroproliferative response (bleomycin-induced lung injury) HSV-TK expression is markedly up-regulated in the injured organ of the mature adult transgenic mice. Compare Western blots of whole lung extracts in Figure 1C (transgenic mouse line 21, bleomycin injured 4-month-old adult mouse) with Figure 9 (uninjured 4-month-old adult mouse). This illustrates that with induction of a fibroproliferative response HSV-TK expression driven by the active type I collagen promoter is up-regulated. Furthermore, HSV-TK expression was selectively up-regulated in the injured lung only. Western analysis of whole organ extracts from other uninjured organs did not show up-regulated HSV-TK expression (data not shown). As supported by our validation study, these data suggest that fibroblasts/myofibroblasts actively forming fibrotic lesions in the localized areas of organ injury will be vulnerable to GCV-mediated ablation.

Discussion

We developed transgenic mice expressing HSV-TK from the type I collagen promoter to evaluate whether the selective elimination of fibroblasts in evolving fibroblastic lesions is an effective therapeutic strategy for fibroproliferative diseases. To validate our transgenic model, we examined the effects of GCV on developing fibrotic tissue in subcutaneously implanted sponges. Our *in vitro* data demonstrate that transgenic sponge fibroblasts/myofibroblasts involved in the fibroproliferative response express HSV-TK and are selectively eliminated by GCV. Consistent with this, our *in vivo* studies indicate that following GCV treatment the development of fibrotic tissue in sponges from transgenic mice is attenuated. This study validates our transgenic model and indicates that this model system is an ideal approach to determine whether ablation of fibroblasts is an effective therapeutic strategy for fibrotic diseases.

HSV-TK Transgenic Murine Model

The HSV-TK transgenic approach has been used to selectively ablate specific cell populations.^{24,30,31,39} This method is based on the selective toxicity of GCV to cells expressing the HSV-TK gene.^{23,40} HSV-TK is capable of phosphorylating specific nucleoside analogs, such as GCV, to nucleoside metabolites that become incorporated into DNA leading to disruption of DNA synthesis and induction of cell death.^{40,41} Two valuable features of this approach include the ability to selectively target a specific cell type by choice of promoter to drive HSV-TK expression and that elimination of the cell type is induc-

ible and can be temporally regulated by when GCV is administered.

We used the HSV-TK transgenic approach to address the question of whether selective ablation of fibroblasts actively forming fibrotic tissue can be used as a therapeutic strategy for fibroproliferative diseases. Fibroblasts/myofibroblasts are the central effector cell resulting in organ fibrosis.^{7,8,10-13} Evidence indicates that myofibroblasts are activated fibroblasts and synthesize high levels of collagen. They constitute the fibroblastic foci of developing fibrotic lesions.^{8,14} Therefore, the cell type we wanted to target for elimination was the fibroblast/myofibroblast. We selected the $\alpha 2$ type I collagen promoter to drive HSV-TK expression and target these cells for elimination because expression is seen only in type I collagen producing cells; this promoter has relatively selective expression by fibroblasts; and it has proven to be successful in driving the expression of reporter genes in transgenic mice.^{42,43} By using the type I collagen promoter to drive HSV-TK expression we selected a promoter that would render fibroblasts actively forming fibrotic lesions susceptible to GCV.

Validation of the Transgenic Model

We chose a sponge implantation approach to examine whether our transgenic model would behave as anticipated. When sponges are implanted subcutaneously they generate an inflammatory and subsequent fibroproliferative response with the formation of fibrotic tissue in the sponges by several weeks. The sponges are then harvested and the cells involved in the fibroproliferative response isolated and characterized. Immunostaining revealed a mixed population of cells isolated from our sponges that included predominantly α -smooth muscle actin- and vimentin-positive cells (determinants characteristic of myofibroblasts) and vimentin- and CD36-positive cells (determinants characteristic of endothelial cells). Although myofibroblasts typically express α -smooth muscle actin and vimentin, other cells such as fibroblasts and mesothelial cells may express these determinants in cell culture. Thus, although the precise identity of these cells is unknown, the morphological and immunohistochemical profile of the cells strongly suggests they are fibroblasts/myofibroblasts. The fibroblasts/myofibroblasts expressed HSV-TK and were vulnerable to GCV-induced killing *in vitro* while endothelial cells did not express HSV-TK and were spared. We also examined our sponges *in vivo* using immunohistochemical techniques to identify the cellular composition of the developing fibrotic tissue. This fibrotic tissue consisted of a population of cells that expressed determinants characteristic of fibroblasts/myofibroblasts (vimentin- and α -smooth muscle actin-positive) and expressed HSV-TK. Consistent with our *in vitro* results, GCV-mediated kill *in vivo* also appeared to be highly selective. We found that the majority of the α -smooth muscle actin-, vimentin-, and HSV-TK-positive cells within these newly formed fibroblastic lesions were selectively targeted for ablation. Semi-quantitative analysis by Western blotting of whole sponge extracts showed

that both α -smooth muscle actin and HSV-TK protein expression virtually disappeared in transgenic mice treated with GCV. Consistent with our *in vitro* studies, our *in vivo* immunohistochemical studies indicate that cells expressing α -smooth muscle actin and vimentin (determinants characteristic of fibroblasts/myofibroblasts) as well as HSV-TK are selectively eliminated by GCV.

An advantage of using the sponge approach to validate our transgenic model is that sponges do not contain collagen. This eliminates the presence of background collagen that could make it difficult to assess the effect of GCV on fibrotic tissue development. Compared to saline, we found that GCV treatment attenuated the development of fibrotic tissue in the sponges, both histologically and biochemically. These results demonstrate that fibroblasts/myofibroblasts derived from sponge fibrotic lesions express HSV-TK and are selectively targeted for GCV-mediated kill *in vitro* and that the development of fibrotic tissue can be attenuated *in vivo*.

The preferential nature by which fibroblasts/myofibroblasts are eliminated in our transgenic mice suggested that non-specific toxicity due to ablation of fibroblasts in other organs may be limited. Consistent with this, after 14 days of GCV treatment, major organs did not display gross or identifiable histological abnormalities although less overt injury remains a possibility. This suggests that without induction of a fibroproliferative response the expression of HSV-TK in fibroblasts present in major organs of mature mice may not be high enough for GCV to have a toxic effect. In support of this, we have found that, in developing mice, HSV-TK expression is present in major organs with the skin, lung, and aorta showing the highest expression. However, in uninjured mature mice the level of expression is much lower with only the lung and skin showing some expression. The relatively sparse nature of fibroblasts populating some of these organs in addition to relatively low type I collagen synthesis in the uninjured state may explain the lack of overt GCV toxicity. Finally, it should be noted that angiogenesis might influence the development of fibrotic tissue in certain fibrotic diseases. In this study we did not examine the effect of GCV on angiogenesis. The purpose of this study was to determine the effect of GCV on fibroblast/myofibroblast removal. Thus, it remains possible that some of the decrease in fibrotic tissue formation by GCV could be due to inhibition of angiogenesis. Additional studies examining the effect of GCV on angiogenesis and wound repair may resolve this issue.

Fibroblast Ablation as a Treatment Strategy for Fibrotic Diseases

We developed this transgenic model to determine whether promoting resolution of physiological fibroproliferation before it becomes pathological by activating apoptosis selectively in fibrotic lesions might be an effective therapeutic strategy for fibrotic diseases. We have demonstrated that the fibroblast/myofibroblast, the primary effector cell of fibrotic disease,^{8,14} is selectively ablated. We anticipate that organ injury in our transgenic mice will

cause the development of a highly concentrated population of myofibroblasts actively producing type I collagen and HSV-TK. Thus these cells should be susceptible to GCV-mediated kill. In support of this, we found that HSV-TK expression in the lung is up-regulated following bleomycin-induced injury (Figure 1C and Reference 44). The major question to be answered is whether ablation of myofibroblasts will have a beneficial effect in terms of limiting fibrosis or whether it will impede repair or worsen injury. We suspect that the timing of GCV-mediated kill during organ repair after injury will be important in determining whether the outcome is favorable in terms of facilitating repair. It is possible that the presence of fibroblasts is essential for parenchymal cell repair and that their premature removal may be detrimental.⁴⁵ An important feature of this transgenic model is that fibroblast kill can be precisely timed by when GCV is administered. This enables us to determine the effects of fibroblast ablation on tissue repair at various times after injury.

Potential Applications of this HSV-TK Transgenic Model

In this report we describe an HSV-TK transgenic murine model that can be used to study the efficacy of ablation of fibroblasts on the development of organ fibrosis in fibroproliferative disease processes that can be modeled in the mouse. Its potential experimental applications are broad and include examining the effect of fibroblast ablation on the development of pulmonary fibrosis after bleomycin or hyperoxic exposures, chemical-induced cirrhosis, cardiac fibrosis, and late remodeling after myocardial infarction or aortic banding, glomerulosclerosis, and renal fibrosis. In addition, the role of fibroblasts during development could also be examined.

Acknowledgments

We thank Drs. Benoit de Crombrughe and Victor Canfield for the type I collagen enhancer/promoter and HSV-TK plasmids, respectively. We also thank Dr. Thomas Wagner for performing the microinjections and Dr. Brett Levay Young for assistance with the sponge experiments.

References

1. Bitterman PB, Henke CA: Fibroproliferative disorders. *Chest* 1991, 99(Suppl 3):81S–84S
2. Basset F, Ferrans VJ, Soler P, Takemura T, Fukuda Y, Crystal RG: Intraluminal fibrosis in interstitial lung disorders. *Am J Pathol* 1986, 122:443–461
3. Ferns GAA, Raines EW, Sprugel KH, Motani AS, Reidy MA, Ross R: Inhibition of neointimal smooth muscle accumulation after angioplasty by an antibody to PDGF. *Science* 1991, 253:1129–1132
4. Gibbons GH, Dzau VJ: The emerging concept of vascular remodeling. *N Engl J Med* 1994, 330:1431–1438
5. Henke C, Fiegel V, Peterson M, Wick M, Knighton D, McCarthy J, Bitterman P: Identification and partial characterization of angiogenesis bioactivity in the lower respiratory tract after acute lung injury. *J Clin Invest* 1991, 88:1386–1395

6. Norman JT, Fine LG: Progressive renal disease: fibroblasts, extracellular matrix, and integrins. *Exp Nephrol* 1999, 7:167–177
7. Svee K, White J, Vaillant P, Jessurun J, Roongta U, Krumwiede M, Johnson D, Henke C: Acute lung injury fibroblast migration and invasion of a fibrin matrix is mediated by CD44. *J Clin Invest* 1996, 98:1713–1727
8. Friedman SL: Molecular regulation of hepatic fibrosis; an integrated cellular response to tissue injury. *J Biol Chem* 2000, 275:2247–2250
9. Hunninghake GW, Kalica AR: Approaches to the treatment of pulmonary fibrosis. *Am J Respir Crit Care Med* 1995, 151:915–918
10. Darby I, Skalli O, Gabbiani G: α -smooth muscle actin is transiently expressed by myofibroblasts during experimental wound healing. *Lab Invest* 1990, 63:21–29
11. Desmouliere A, Redard M, Darby I, Gabbiani G: Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar. *Am J Pathol* 1995, 146:56–66
12. Fukuda Y, Ishizaki M, Musada Y, Kimuri G, Kawanami O, Masugi Y: The role of intra-alveolar fibrosis in the process of pulmonary structural remodeling in patients with diffuse alveolar damage. *Am J Pathol* 1987, 126:171–182
13. Zeisberg M, Strutz F, Muller GA: Role of fibroblast activation in inducing interstitial fibrosis. *J Nephrol* 2000, 13(Suppl 3):S111–S120
14. Kuhn C, McDonald JA: The roles of the myofibroblast in idiopathic pulmonary fibrosis. *Am J Pathol* 1991, 138:1257–1265
15. Arends MJ, Wyllie AH: Apoptosis: mechanisms and roles in pathology. *Int Rev Exp Pathol* 1991, 32:223–254
16. Ellis RE, Yuan JY, Horvitz HR: Mechanisms and functions of cell death. *Annu Rev Cell Biol* 1991, 7:663–698
17. Kapanci Y, Weibel ER, Kaplan HP, Robinson FR: Pathogenesis and reversibility of the pulmonary lesions of oxygen toxicity in monkeys. *Lab Invest* 1969, 20:101–118
18. Meduri GU, Belenchia JM, Estes RJ, Wunderink R, Eltorkey M, Leeper KV: Fibroproliferative phase of ARDS: clinical findings and effects of corticosteroids. *Chest* 1991, 100:943–952
19. Meduri GU, Chinn AJ, Leeper KV, Wunderink RG, Tolley E, Winer-Muram HT, Khare V, Eltorkey M: Corticosteroid rescue treatment of progressive fibroproliferation in late ARDS: patterns of response and predictors of outcome. *Chest* 1994, 105:1516–1527
20. Polunovsky VA, Chen B, Henke C, Snover D, Wendt C, Ingbar DH, Bitterman PB: Role of mesenchymal cell death in lung remodeling after injury. *J Clin Invest* 1993, 92:388–397
21. Suchyta MR, Elliott CG, Colby T, Rasmussen BY, Morris AH, Jensen RL: Open lung biopsy does not correlate with pulmonary function after the adult respiratory distress syndrome. *Chest* 1991, 99:1232–1237
22. Tian B, Lessan K, Kahm J, Kleidon J, Henke C: β 1 integrin regulates fibroblast viability during collagen matrix contraction through a phosphatidylinositol 3-kinase/Akt/protein kinase B signaling pathway. *J Biol Chem* 2002, 277:24667–24675
23. Borrelli E, Heyman R, His M, Evans RM: Targeting of an inducible toxic phenotype in animal cells. *Proc Natl Acad Sci USA* 1988, 85:7572–7576
24. Borrelli E, Heyman RA, Arias C, Sawchenko PE, Evans RM: Transgenic mice with inducible dwarfism. *Nature* 1989, 339:538–541
25. Burkhardt A: Alveolitis and collapse in the pathogenesis of pulmonary fibrosis. *Am Rev Respir Dis* 1989, 140:513–524
26. Goldstein RH: Control of type I collagen formation in the lung. *Am J Physiol* 1991, 261:L29–L40
27. Kuhn III C, Boldt J, King Jr TE, Couch E, Vartio T, McDonald JA: An immunohistochemical study of architectural remodeling and connective tissue synthesis in pulmonary fibrosis. *Am Rev Respir Dis* 1989, 140:1693–1703
28. Laurent GJ: Dynamic state of collagen: pathways of collagen degradation in vivo and their possible role in regulation of collagen mass. *Am J Physiol* 1987, 252:C1–C9
29. Lazenby AJ, Crouch EC, McDonald JA, Kuhn III C: Remodeling of the lung in bleomycin-induced pulmonary fibrosis in the rat. *Am Rev Respir Dis* 1990, 142:206–214
30. Wallace H, Ledent C, Vassart G, Bishop JO, Al-Shawi R: Specific ablation of thyroid follicle cells in adult transgenic mice. *Endocrinology* 1991, 129:3217–3226
31. Bush TG, Savidge TC, Freeman TC, Cox HJ, Campbell EA, Mucke L, Johnson MH, Sofroniew MW: Fulminant jejuno-ileitis following ablation of enteric glia in adult transgenic mice. *Cell* 1998, 93:189–201

32. Chen B, Polunovsky V, White J, Blazar B, Nakhleh R, Jessurun J, Peterson M, Bitterman P: Mesenchymal cells isolated after acute lung injury manifest an enhanced proliferative phenotype. *J Clin Invest* 1992, 90:1778–1785
33. Henke CA, Roongta U, Mickelson DJ, Knutson JR, McCarthy JB: CD44-related chondroitin sulfate proteoglycan, a cell surface receptor implicated with tumor cell invasion, mediates endothelial cell migration on fibrinogen and invasion into a fibrin matrix. *J Clin Invest* 1996, 97:2541–2552
34. Tian B, Carlyle WC, Weigold WG, McDonald K: M, Judd DL, Toher CA, Homans DC, Cohn JN: Localization of changes in β -actin expression in remodeled canine myocardium. *J Mol Cell Cardiol* 1999, 31:751–760
35. Tian B, Liu L, Bitterman PB, Bache RJ: Mechanisms of cytokine induced NO-mediated cardiac fibroblast apoptosis. *Am J Physiol* 2002, 283:H1958–H1967
36. Berg RA: Determination of 3- and 4- hydroxyproline. *Methods Enzymol* 1982, 82:372–398
37. Mukherjee D, Sen S: Collagen phenotypes during development and regression of myocardial hypertrophy in spontaneously hypertensive rats. *Circ Res* 1990, 67:1474–1480
38. Al-Shawi R, Burke J, Jones CT, Simons JP, Bishop JO: A MUP promoter-thymidine kinase reporter gene shows relaxed tissue-specific expression and confers male sterility upon transgenic mice. *Mol Cell Biol* 1988, 8:4821–4828
39. Canfield V, West AB, Goldenring JR, Levenson R: Genetic ablation of parietal cells in transgenic mice: a new model for analyzing cell lineage relationships in the gastric mucosa. *Proc Natl Acad Sci USA* 1996, 93:2431–2435
40. Heyman RA, Borrelli E, Lesley J, Anderson D, Richman DD, Baird SM, Hyman R, Evans RM: Thymidine kinase obliteration: creation of transgenic mice with controlled immune deficiency. *Proc Natl Acad Sci USA* 1989, 86:2698–2702
41. Frank KB, Chiou JF, Cheng YC: Interaction of herpes simplex virus-induced DNA polymerase with 9-(1, 3-dihydroxy-2-propoxymethyl) guanine triphosphate. *J Biol Chem* 1984, 259:1566–1569
42. Bou-Gharios G, Garrett LA, Rossert J, Niederreither K, Eberspaecher H, Smith C, Black C, de Crombrughe B: A potent far-upstream enhancer in the mouse $\text{pro}\alpha 2(\text{I})$ collagen gene regulates expression of reporter genes in transgenic mice. *J Cell Biol* 1996, 134:1333–1344
43. Chen SS, Ruteshouser EC, Maity SN, de Crombrughe B: Cell-specific in vivo DNA-protein interactions at the proximal promoters of the $\text{pro}\alpha 1(\text{I})$ and the $\text{pro}\alpha 2(\text{I})$ collagen genes. *Nucleic Acids Res* 1997, 25:3261–3268
44. Han L, Tian B, Kleidon J, Henke C: Fibroblast ablation as treatment for pulmonary fibrosis. *Am J Respir Crit Care Med* 2002, 165:A365 (Abstract)
45. Panos RJ, Rubin JS, Aaronson SA, Mason RJ: Keratinocyte growth factor and hepatic growth factor/scatter factor are heparin-binding growth factors for alveolar type II cells in fibroblast-conditioned medium. *J Clin Invest* 1993, 92:969–977