UNIVERSIDAD AUTONOMA DE NUEVO LEON
FACULTAD DE MEDICINA

EFECTIVIDAD DEL SISTEMA *HSV-tk*/GCV MEDIADO POR TERAPIA GENICA EN TUMORES MAMARIOS ORTOTOPICOS Y HETEROTOPICOS

POR

MCP Y MC AUGUSTO ROJAS MARTINEZ

Como requisito parcial para obtener el Grado de
DOCTOR EN CIENCIAS con Especialidad en Biología
Molecular e Ingeniería Genética

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El trabajo experimental de esta tesis se desarrolló en el Laboratorio de Vectores de Terapia Génica, en el Centro para Terapia Celular y Génica, del Baylor College of Medicine, en Houston, Texas; bajo la dirección del ***Dr. Carlos Estuardo Aguilar Córdova****, y siguiendo la normatividad del programa de posgrado del que forma parte el Doctorado en Ciencias con Especialidad en Biología Molecular e Ingeniería Genética, de la UANL,*
el ***Dr. Hugo A. Barrera Saldaña*** *fungió como co-director.*

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A Mamá y a mis hermanos.

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NOMENCLATURA

ADN	Ácido Desoxirribonucleico
AdV-CMV-βgal	Vector adenoviral portando el gen *Lac Z*
Adv-RSV-tk	Vector adenoviral portando el gen *timidin cinasa*
α-MEM	Medio esencial mínimo tipo alfa.
ANOVA	Análisis de varianza (en inglés)
BALB/c	Cepa endogámica de ratón albino
βgal o β-gal	Gen de β-galactosidasa (gen *LacZ*)
C57BL/6	Cepa endogámica de ratón negro
cm^2	Centímetros cuadrados
cm^3	Centímetros cúbicos
CO_2	Bióxido de carbono
cols.	Colaboradores
cpm	Cuentas por minuto
^{51}Cr	Isótopo de cromo de peso atómico 51
DP	Diámetro promedio
$DP_{dia\ 0}$	Diámetro promedio al día de la transducción adenoviral
$DP_{dia\ X}$	Diámetro promedio a cualquier día de observación
E	10 exponente (Logaritmo base 10)
EUA	Estados Unidos de América
FSK	Línea celular de epitelio mamario murino (cepa BALB/C)
g	Gramos
°C	Grados centígrados
GCV	Ganciclovir
h	Horas
HCMVIE (ó CMV)	Promotor temprano del citomegalovirus humano
HeLa	Línea celular humana de cáncer de cuello uterino
HSV-tk	Gen timidin cinasa del virus herpes simplex tipo I
ICR	Indice de crecimiento relativo
IL-2	Interleucina 2
kg	Kilogramo
kpb	miles de pares de bases
LCT	Linfocitos citotóxicos
mg	Miligramos
min	Minutos
ml	Mililitros
mM	Milimolar
mm^3	Milímetros cúbicos
μCi	MicroCuries
μg	Microgramos
μm	Micrómetros
ng	Nanogramos
μl	Microlitros
nm	Nanómetros.
p	Valor de probabilidad

p.v.	Partículas de vector
PBS	Solución salina de fosfatos (en inglés)
pH	Potencial de hidrogeniones
PNCT	Proporción neta de crecimiento tumoral
%	Por ciento
REDOX	Agente óxido-reductor
RPMI 1640	Medio de cultivo para leucocitos
RSV	Promotor del virus del sarcoma de Rous
SV_{50}	Dosis con sobrevida de 50 por ciento
$TCLD_{50}$	Dosis de cultivo de tejidos con sobrevida de 50 por ciento (en inglés)
TM40D	Línea celular de cáncer mamario murino (cepa BALB/C)
U	Unidades
u.m.	Unidad de mapa del genoma de adenovirus (36 kpb = 100 u.m.)
x	Veces ó multiplicación
xg	Gravedades (Centrifugación)

RESUMEN

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Universidad Autónoma de Nuevo León
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Fecha de Graduación. Mayo del 2000

Título del Estudio: EFECTIVIDAD DEL SISTEMA *HSV-tk*/GCV MEDIADO POR TERAPIA GÉNICA EN TUMORES MAMARIOS ORTOTÓPICOS Y HETEROTÓPICOS

Número de páginas: 49

Candidato para el grado de Doctor en Ciencias con especialidad en Biología Molecular e Ingeniería Genética.

Propósito y método del estudio: El advenimiento de la terapia génica ha generado una nueva línea de investigación en la lucha contra el cáncer. En la actualidad hay decenas de ensayos clínicos explorando esta modalidad terapéutica en contra de diversos tipos de tumores. El análisis de los tratamientos para el cáncer, como los medicamentos quimioterapéuticos, generalmente se inicia con un estudio en modelos preclínicos que incluyen estudios en cultivos celulares y en modelos animales. En cuanto a los modelos animales, los tumores generados para las pruebas pueden implantarse en el órgano de donde deriva originalmente el tumor (modelo ortotópico), pero debido a dificultades técnicas, muchas veces los tumores son implantados en un sitio más accesible para determinar la evolución y los efectos del tratamiento (modelo heterotópico), como el espacio subcutáneo. Sin embargo, factores del microambiente que rodea la neoplasia pueden generar diferencias en la efectividad del agente antineoplásico. Esta posibilidad no había sido examinada hasta la fecha en los estudios preclínicos de terapia génica.

En este estudio se analizaron los efectos del microambiente en la respuesta tumoral a la terapia génica con el sistema de toxicidad condicionada denominado timidín quinasa del virus herpes simplex I/ganciclovir (*HSV-tk*/GCV), en un modelo murino implantado con células singénicas de tumor mamario TM40D. Las células se implantaron en el espacio subcutáneo (modelo heterotópico) y en la glándula mamaria número 4 (modelo ortotópico) de ratonas BALB/c. Estos sitios, aunque están relacionados morfológica y embriológicamente, tienen características fisiológicas diferentes. Los animales fueron separados en dos grupos: Un grupo de tratamiento tratado con el sistema *HSV-tk*/GCV y un grupo de control para efectos del vector con el gen *lac Z* de Escherichia coli que es farmacológicamente inactivo (β-gal/GCV). Se analizaron las curvas de crecimiento de los tumores durante el tratamiento y la sobrevida posterapeútica en los dos modelos experimentales. Una vez sacrificadas las ratonas, se estudió la histología de los tumores y la respuesta citotóxica de linfocitos extraídos del bazo en contra de las células implantadas.

Contribuciones y conclusiones: Aunque se observó una supresión transitoria del crecimiento tumoral en ambos modelos de implantación (33.1 y 39.4% en los modelos ortotópico y heterotópico, respectivamente) la tasa de crecimiento tumoral y la respuesta a la terapia fueron significativamente diferentes. Histológicamente, los dos modelos mostraron patrones de necrosis, vascularidad y respuesta tisular inmune diferentes. La respuesta citotóxica linfocitaria y la sobrevida no mostraron diferencias significativas. Los datos sugieren que el microambiente del tumor puede afectar la eficacia de la terapia génica.

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CAPÍTULO I

INTRODUCCIÓN

La búsqueda de mejores tratamientos para los tumores malignos impulsa la investigación de nuevos medicamentos, combinaciones de agentes quimioterapéuticos, incrementos de dosis, utilización de citocinas, regímenes mejorados de radioterapia y cirugías más sofisticadas, entre otros recursos. Sin embargo estas formas de tratamiento no superan algunos obstáculos biológicos críticos, como la actividad localizada en el tumor o la carencia de efectos en células normales, y no podrían conducir a un avance radical en el mejoramiento del tratamiento del cáncer. La terapia génica ofrece el potencial para superar algunas de esas barreras fundamentales y para disminuir la morbilidad asociada al tratamiento.

La terapia génica puede definirse como la modalidad de tratamiento en la cual se introduce material genético a las células para modificarlas o añadirles nuevas funciones. Este tipo de terapia involucra la transferencia de material genético foráneo a las células con el propósito de corregir o aminorar los defectos genéticos en algunas enfermedades hereditarias, inhibir agentes

infecciosos o destruir células cancerosas (Eck y Wilson, 1996). De forma muy general, la terapia génica requiere la identificación y aislamiento de genes terapéuticos útiles y la transferencia y expresión subsecuente de estos genes en las células receptoras o células blanco (Bank A, 1995). En la actualidad la terapia génica está siendo ensayada para el tratamiento de una amplia gama de enfermedades hereditarias y adquiridas, entre ellas el cáncer (Eck y Wilson, 1996).

A continuación se describen las ventajas potenciales de la terapia génica antineoplásica. En las terapias convencionales, como la quimioterapia, una célula o un tejido se altera con la modificación del metabolismo y de la fisiología celular al nivel de la expresión de proteínas. En contraste, en la terapia génica, el cambio en el patrón de expresión de genes en las células transducidas podría modificar la cantidad y la función de los productos proteicos preexistentes o la generación de nuevas proteínas para lograr el efecto terapéutico deseado. Adicionalmente, la transferencia génica se puede realizar en un contexto limitado local-regional, produciendo una alta concentración de moléculas terapéuticas en el área de interés, evitando la difusión del agente activo al sistema y disminuyendo los efectos indeseables de los agentes convencionales.

Otra ventaja de la terapia génica es su potencial para lograr una actividad terapéutica de más alta especificidad de acción que la obtenida con la

quimioterapia, gracias al control de los mecanismos regulatorios de la expresión génica. Por otro lado, la existencia de diversos sistemas de transferencia de genes (vectores) para la modificación genética de largo plazo o permanente de células o tejidos, permitiría intervenciones terapéuticas o preventivas definitivas (Eck y Wilson, 1996). Finalmente, el emplear al organismo para producir proteínas potencialmente terapéuticas en solo ciertos tejidos, tiene ventajas prácticas, pues se evitan las limitaciones asociadas con la manufactura, estabilidad y duración del efecto, después de la administración de los medicamentos (Eck y Wilson, 1996).

El cáncer surge como la culminación de una sucesión de pasos que involucran alteraciones de diferentes genes de las células somáticas y modificaciones importantes en el microambiente que rodea al tumor. Por estas razones, esta enfermedad representa un gran reto para la terapia génica, pues es difícil hacer una elección del gen terapéutico ideal para lograr la destrucción del tejido neoplásico (Rabkin y cols., 1996) y por esto se han generado varias estrategias de terapia génica antitumoral.

Las estrategias de terapia génica antineoplásica se pueden agrupar en las siguientes categorías: inmunopotenciación, corrección fenotípica (inactivación de oncogenes o reemplazo de genes supresores de tumor), la quimioterapia molecular, el incremento de la resistencia a drogas para proteger la médula ósea de altas dosis de quimioterapia y esquemas de terapia

potenciadora, en la cual la terapia génica sensibiliza a las células tumorales para ser destruidas en forma más eficiente por un agente antineoplásico o por radioterapia (Cusack y Tanabe, 1998).

La terapia génica suicida (también denominada terapia de citotoxicidad condicionada) ha sido extensamente investigada para el tratamiento de tumores primarios y diseminados. Entre los diversos sistemas de esta modalidad, el esquema de la enzima timidín cinasa del virus herpes simplex (*HSV-tk*) y la subsecuente administración de ganciclovir (GCV) ha sido exitoso en el tratamiento de tumores en modelos animales de cáncer refractario a las modalidades terapéuticas convencionales en humanos (Culver y cols., 1992 y Chen y cols., 1994) y por otro lado, ha mostrado tener una baja toxicidad sistémica (Rojas-Martínez y cols., 1999). Esta estrategia se basa en la conversión del GCV (un análogo de nucleósidos purínicos) en su intermediario activo, GCV monofosfato, en células transducidas con el gen *HSV-tk*. La prodroga fosforilada conduce a la muerte celular al detener la síntesis de ADN (Reid y cols., 1988). Adicionalmente, las células no transducidas son afectadas por la exportación de la sustancia activa (efecto testigo) (Freeman y cols., 1993) o por la estimulación del sistema inmune en respuesta a las células afectadas por el sistema *HSV-tk*/GCV (Barba y cols. 1994 y Yamamoto y cols. 1997). Debido a que la respuesta terapéutica depende de varios mecanismos, la efectividad puede ser susceptible a las diferencias en el microambiente tumoral.

Los modelos animales experimentales para probar tratamientos antineoplásicos se pueden dividir en dos grupos, de acuerdo al sitio anatómico de donde se deriva el tumor que se desea estudiar. En el modelo ortótopico, el tumor se genera en el tejido u órgano de donde deriva la malignidad a analizar (por ejemplo, implantación de hepatoma en el hígado). En el modelo heterotópico, el tumor se crea en un espacio u órgano diferente al sitio donde se genera el tumor en forma natural (por ejemplo, la implantación subcutánea de tumores de diverso origen diferentes a los cánceres que afectan la piel y el espacio dérmico). La elección del modelo a utilizar frecuentemente se basa en la disponibilidad de tumores o líneas celulares y animales, y en la conveniencia del investigador.

En la terapia génica para el cáncer se han empleado varios modelos animales para evaluar la efectividad de la terapia con el sistema *HSV-tk*/GCV. En algunos casos, animales implantados con tumores subcutáneos de próstata (Eastham y cols. 1996) y mesotelioma (Smythe y cols., 1995), buenos ejemplos de modelos heterotópicos, han sido tratados con este sistema. Glioblastomas implantados en cerebro (Culver y cols., 1992 y Chen y cols., 1994) o tumores primarios de glándula mamaria inducidos por transgénesis (Sacco y cols., 1996) o sustancias químicas (Wei y cols., 1998), han sido ejemplos de modelos ortotópicos tratados con el sistema *HSV-tk*/GCV. Tanto los modelos heterotópicos como ortotópicos han generado resultados exitosos que han sido utilizados como base para la implementación de estudios de terapia génica con

el sistema *HSV-tk*/GCV en fase clínica. Por ejemplo, los resultados favorables obtenidos con el sistema *HSV-tk*/GCV en los estudios de tumores cerebrales en rata (Ram y cols., 1993), sirvieron para sustentar un ensayo en fase clínica I para el tratamiento de glioblastoma (Ram y cols., 1997), el cual demostró actividad antitumoral en 5 de 15 pacientes con tumores de menos de 1.4 ml de volumen. También se pueden mencionar los estudios de Eastham y cols. En cáncer de próstata (Eastham y cols., 1996), que sirvieron de base a estudios de fase clínica I en pacientes con recurrencia de este tipo de tumor después de radioterapia y que mostró respuestas positivas en 3 de los 18 sujetos seleccionados. Esta respuesta fue evidenciada por una caída de los niveles de antígeno prostático específico que fue sostenida durante un periodo de un año (Herman y cols. 1999).

Sin embargo, los efectos del microambiente tumoral en el modelo preclínico pueden afectar significativamente el valor pronóstico de los estudios clínicos y estos efectos no han sido estudiados aun en este tipo de terapia. Entre los factores microambientales que afectarían la efectividad terapéutica están la arquitectura tisular, la respuesta local del tejido, el patrón de vascularización y la vigilancia inmunológica. El propósito de esta tesis es estudiar la eficacia de la estrategia del sistema *HSV-tk*/GCV en los microambientes ortotópico y heterotópico, usando un modelo preclínico de cáncer mamario.

CAPITULO II

HIPÓTESIS Y OBJETIVOS

2.1 HIPÓTESIS.

Los efectos de la terapia génica para el cáncer con el sistema *HSV-tk*/GCV, pueden variar en diferentes microambientes tumorales. Es posible que la respuesta antineoplásica para los tumores mamarios TM40D cambie en los diferentes sitios de implantación tumoral del modelo animal.

2.2. OBJETIVOS

2.2.1. Objetivo General:

Analizar la eficacia del sistema suicida HSV-tk/GCV en tumores mamarios TM40D de localización ortotópica y heterotópica en un modelo murino.

2.2.2. Objetivos Específicos:

1. Determinar la sensibilidad de las células TM40D al sistema *HSV-tk*/GCV en condiciones de cultivo de tejidos.

2. Determinar el crecimiento tumoral natural de tumores TM40D implantados en los modelos orto y heterotópico.

3. Hacer un escalamiento de dosis de vector para determinar la dosis antitumoral más efectiva.

4. Determinar los efectos del sistema *HSV-tk*/GCV en los modelos orto y heterotópico, mediante el análisis de los siguientes parámetros obtenidos de la observación de los dos grupos experimentales (ratonas tratadas con el gen *HSV-tk* y GCV como grupo de tratamiento y ratonas tratadas con el gen β-gal y GCV como grupo control):

   a. Curva de crecimiento tumoral.
   b. Análisis histopatológico.
   c. Análisis de linfocitos citotóxicos (LCT).
   d. Supervivencia.

CAPÍTULO III

ESTRATEGIA GENERAL

Los parámetros claves de la tesis se estudiaron con las siguientes estrategias:

3.1. SUSCEPTIBILIDAD DE LA LÍNEA TUMORAL TM40D AL SISTEMA *HSV-tk*/GCV. La susceptibilidad de la línea tumoral mamaria TM40D al vector adenoviral y al sistema de toxicidad condicionada *HSV-tk*/GCV fue probada en condiciones de cultivo de tejidos, en un ensayo comparativo realizado en un formato de 8 x 12 pozos y en el cual se usaron células HeLa como control. Las células fueron transducidas con dosis incrementadas del vector adenoviral Adv-RSV-*tk* y tratadas con GCV. Tres días después del tratamiento se hizo un ensayo colorimétrico con un agente REDOX para determinar la actividad metabólica y calcular la sobrevida de las células. Una vez demostrada la susceptibilidad de las células cancerosas al vector y al sistema suicida, se procedió a los estudios *in-vivo* en modelos murinos.

3.2. DETERMINACIÓN DEL CRECIMIENTO NATURAL DE LOS IMPLANTES TUMORALES ORTOTÓPICOS Y HETEROTÓPICOS: Para determinar el efecto terapéutico en las ratonas, se establecieron curvas de crecimiento de referencia en animales implantados en el espacio subcutáneo (heterotópico) y en la glándula mamaria número 4 (ortotópico).

3.3. ESCALAMIENTO DE DOSIS: Como en el experimento anterior se determinó que el crecimiento era más agresivo en el espacio ortotópico, el escalamiento de la dosis de vector más efectiva se realizó en este modelo. El objetivo de este experimento fue determinar las dosis tóxicas del vector y la dosis máxima tolerada que tuviera efecto antineoplásico. Las ratonas fueron tratadas durante 14 días consecutivos con GCV y se realizaron curvas de crecimiento tumoral para determinar la dosis más efectiva.

3.4. EFICACIA DEL SISTEMA *HSV-tk*/GCV EN LOS MODELOS ORTO Y HETEROPTÓPICO. La línea tumoral TM40D se implantó en dos grupos de ratonas: Uno en el espacio subcutáneo y el otro en la glándula mamaria. Cuando el crecimiento de los tumores permitió la manipulación experimental, los animales en cada modelo de implantación fueron divididos en dos grupos: Un grupo de ratonas recibió una inyección intratumoral con el vector adenoviral Adv-RSV-*tk* y al grupo restante se le administró el vector AdV-CMV-βgal, no funcional desde el punto de vista terapéutico, pero apto para servir como control del grupo de tratamiento. Todas las ratonas fueron tratadas con GCV durante los 14 días subsecuentes a la transducción y se realizaron curvas de crecimiento tumoral. Una vez concluida la observación, las ratonas se sacrificaron, los tumores y algunos de los bazos se disecaron y extrajeron para análisis de patología y para realizar pruebas de linfocitos citotóxicos anti-TM40D.

3.5. ANÁLISIS DE SOBREVIDA. En este estudio, se repitió el experimento anteriormente descrito, pero los animales no se sacrificaron y se determinó la sobrevida de cada animal hasta que estuviera en muy malas condiciones generales o cuando el tumor alcanzaba un diámetro mayor a 2.5 cm, difícil de ser tolerado por la ratona. Con los datos de sobrevida de

cada animal se realizó una curva de Kaplan-Meyer para analizar la eficacia de la terapia génica para prolongar la sobrevida en cada modelo de implantación.

3.6. PRUEBA DE LINFOCITOS CITOTÓXICOS ANTI-TM40D. Para determinar la generación de respuesta celular inmune inducida por la terapia génica en contra de la línea tumoral, los esplenocitos extraídos de los bazos fueron aislados y cultivados en medio RPMI 1640 enriquecido para seleccionar la población de linfocitos citotóxicos. Después de la cosecha, los linfocitos obtenidos se mezclaron en diferentes proporciones con células blanco TM40D mantenidas en cultivo. Estas células blanco habían sido previamente irradiadas y tratadas con ^{51}Cr. Después de un breve tiempo de cocultivo, los sobrenadantes recolectados se analizaron para determinar la actividad citotóxica mediante la medición de la radiactividad presente en el medio recolectado.

CAPÍTULO IV

MATERIALES Y MÉTODOS

4.1. ORIGEN DE LOS REACTIVOS:

4.1.1. Células Tumorales: La línea celular de tumor mamario de ratón TM40D fue derivada de transplantes repetidos de las líneas celulares de mama FSK en glándulas mamarias de ratonas BALB/c (Kittrell y cols., 1992). Las células se mantuvieron en condiciones de cultivo utilizando medio α-MEM (Life-Technologies, Gaithersburg, MD, EUA) suplementado con suero bovino de ternera al 10% (HyClone, Logan, UT, EUA) a temperatura de 37°C y una atmósfera con 5% de CO_2. Para la implantación en el modelo *in-vivo*, las células fueron recolectadas de cultivos en monocapa mediante un tratamiento con tripsina al 0.25% (Life-Technologies, Gaithersburg, MD, EUA), lavado con solución amortiguadora de fosfatos (PBS por las siglas en inglés y cuya composición es: 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_20$ y 1.4 mM KH_2PO_4, pH ~ 7.3), cosecha, centrifugación a 500 xg durante 2.5 min, cuantificación y resuspensión final en PBS.

4.1.2. Vectores adenovirales: Se utilizaron vectores adenovirales portando un gen terapéutico (efector) y un gen control. El vector efector ADV-RSV-*tk* (ver figura 1) contiene el gen *HSV-tk* del virus herpes simplex tipo I con la secuencia promotora de la repetición terminal larga del virus del sarcoma de Rous (RSV), contenidas ambas dentro del esqueleto genómico del

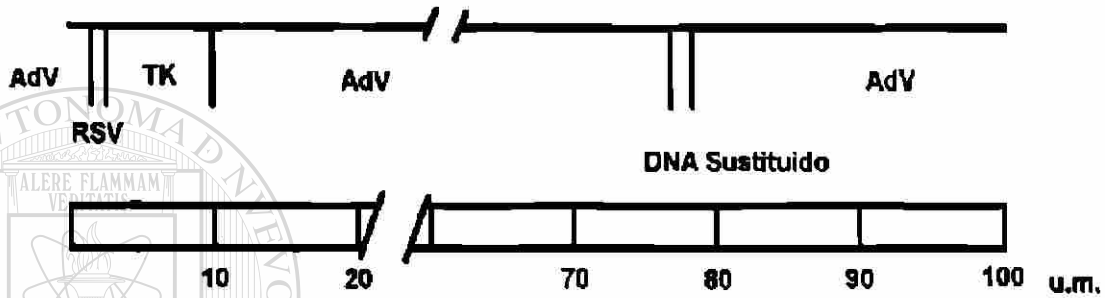

Figura 1. Genoma del vector AdV-RSV-*tk*. Representación de la estructura genómica del vector terapéutico utilizado. El gen terapéutico *HSV-tk* sustituye las regiones requeridas para la replicación adenoviral E1A y E1B. El gen está regulado por la secuencia promotora del virus del Sarcoma de Rous (RSV). El genoma del vector AdV, de 36 kpb, se divide convencionalmente en 100 unidades de mapa (u.m.).

adenovirus humano tipo 5 (Chen y cols., 1994). El vector control AdV-CMV–β-gal tiene el mismo esqueleto genómico, pero en lugar del gen *HSV-tk*, contiene el gen *lac-Z* dirigido por el promotor inmediato-temprano del citomegalovirus humano (HCMVIE). Los vectores fueron producidos y cuantificados en el Laboratorio de Vectores de Terapia Génica del Baylor College of Medicine en Houston, TX, EUA (Nyberg-Hoffman y cols., 1997).

4.2. MÉTODOS:

4.2.1. Susceptibilidad de la línea tumoral TM40D al sistema *HSV-tk*/GCV. Se realizó un ensayo comparativo en un formato de 8 x 12 pozos (Corning Inc., Corning, NY, EUA) y en el cual se usaron células HeLa (ATCC, Rockville, MD, EUA) como control. Las células TM40D y las células control se sembraron en formatos separados a una densidad de 3 x 10^4 células/cm^2 (confluencia de aproximadamente 50%). Las columnas fueron transducidas con dosis incrementadas del vector adenoviral AdV-RSV-*tk* en forma aritmética, abarcando un rango de ~5 x 10^5 a 3 x 10^9 p.v./ml (razón máxima: 30,000 p.v./célula). Después de 12 horas de la siembra, el formato fue dividido en dos áreas de tres filas. El área superior fue tratada con la forma comercial de GCV, Cimevene® (Sintex Corporation, Palo Alto, CA, EUA), utilizada en todos los estudios de este proyecto; mientras que la zona inferior de tres filas fue tratada con medio de cultivo α-MEM suplementado con suero bovino de ternera al 10%. La primera columna de células no fue tratada con el vector ni con GCV, para servir como referencia de máxima sobrevida y sólo fue adicionada con medio de cultivo cada vez que se hacía un tratamiento. Tres días después del tratamiento con GCV, se hizo un ensayo colorimétrico con el agente REDOX Alamar Blue® (Alamar Biosciences Inc., Sacramento, CA, EUA), que determina la actividad metabólica en cada pozo de células y permite determinar la supervivencia en cada columna (Lancaster y Fields, 1996). Un área de 3 pozos en el margen del formato sólo se había llenado con

medio para servir como blanco de lectura del experimento después de la adición del agente REDOX. La absorbancia se midió en un aparato para análisis de ELISA tipo Microplate Autoreader® (Bio-Tek Instruments Inc., Burlington, VE, EUA) a una longitud de onda de 570 nm con referencia a 600 nm. Los datos de absorbancia de los dos tipos celulares tratados se determinaron en triplicado, fueron promediados y se determinó el error estándar para cada medición. El índice de sobrevida se calculó dividiendo la absorbancia de cada dosis probada entre la absorbancia obtenida en las células no tratadas con el vector ni con GCV. Los índices obtenidos fueron utilizados para la construcción de la gráfica de susceptibilidad al vector adenoviral y al sistema *HSV-tk*/GCV en condiciones *in-vitro*. Las dosis letales 50 en cultivo ($TCLD_{50}$ en inglés), la dosis en la cual el 50% de las células aun se mantienen vivas, fue calculada para cada línea celular a partir de la gráfica.

4.2.2. Implantación de los tumores ortotópicos y heterotópicos. Tumores orto y heterotópicos fueron generados en 238 ratonas vírgenes BALB/c de 5 y 6 semanas de edad (Harlan Inc., Indianápolis, IN, EUA). En 147 ratonas se generaron tumores ortotópicos y en las restantes 91 se produjeron tumores heterotópicos. Adicionalmente, se implantaron tumores heterotópicos en 5 ratones machos C57BL/6 (Harlan Inc., Indianápolis, IN, EUA) para los ensayos de linfocitos citotóxicos. Para los procedimientos de implantación, las ratonas fueron sometidas a anestesia

general mediante inyección intraperitoneal de pentobarbital (Nembutal®, Abbott Laboratories, Chicago, IL, EUA) a una dosis de 50 mg/kg de peso.

En el caso de los tumores ortotópicos, a las ratonas se les practicó una incisión paramedial en el hemiabdomen izquierdo seguida por una incisión inguinal. Posteriormente se hacía una disección cuidadosa del tejido subcutáneo para exponer la glándula mamaria número 4. A continuación, la glándula mamaria fue inyectada con un volumen de 20 µl que contenía un total de 5,000 células TM40D suspendidas en PBS (figura 2). Las inyecciones se realizaron con una jeringa graduada en microlitros (Hamilton Co., Reno, NV, EUA) utilizando una aguja 27-G.

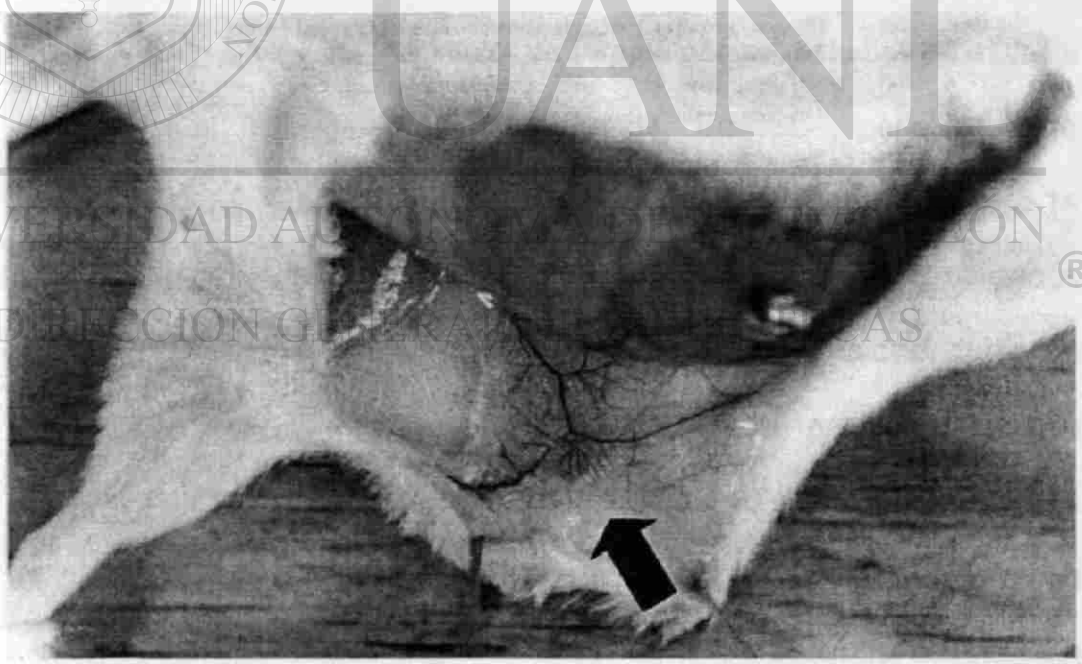

Figura 2. Cirugía para la implantación del tumor en la glándula mamaria número 4: La glándula está localizada en el flanco inferior abdominal, cerca de la región inguinal. El parénquima glándular está albergado en el interior del cojinete de tejido graso (señalado por la flecha), adherido al tejido subcutáneo. Obsérvese la rica vascularización del órgano.

Después de la inyección, se afrontaron los planos y se cerraron las heridas quirúrgicas con ganchos Autoclip® de 9 mm (Clay Adams, Sparks, MD, EUA). En el caso de los tumores heterotópicos, las ratonas anestesiadas fueron afeitadas en el flanco dorsal izquierdo y en el espacio subcutáneo de esta porción se realizó una inyección con el mismo número de células usado en el modelo ortotópico. Estas células fueron inyectadas en un volumen de 50 µl, utilizando jeringas desechables de 1 ml adaptadas con una aguja 30-G.

4.2.3. Administración del vector de terapia génica y tratamiento con GCV. Este tipo de tratamientos se realizó en tres fases experimentales: Escalamiento de la dosis del vector de terapia génica, estudios de eficacia antineoplásica y análisis de la sobrevida. En los experimentos de escalamiento de la dosis de vector se utilizaron 47 ratonas, incluyendo 14 animales cuyos tumores fueron inyectados con PBS para ser utilizados como control de no tratamiento. Los experimentos de escalamiento de dosis para AdV-RSV-*tk* fueron realizados en el modelo ortotópico utilizando un rango de dosis entre 1 x 10^9 a 1 x 10^{11} p.v., incrementadas en una escala logarítmica decimal (dosis por encima de 1 x 10^{12} p.v. son tóxicas para este roedor, según la observación personal del tesista). En las fases de eficacia antineoplásica y análisis de la sobrevida, se utilizaron los vectores AdV-RSV-*tk* (vector terapéutico) y AdV-CMV-βgal (vector

control). Todos los vectores fueron administrados mediante inyección intratumoral de un bolo de 20 µl de suspensión del vector, aproximadamente dos semanas después de la implantación tumoral (volumen tumoral promedio de 40 mm^3). En el caso del modelo ortotópico, la administración del vector requirió el mismo procedimiento quirúrgico descrito para la implantación del tumor.

El tratamiento con GCV se inició 24 h después de la administración de los vectores. Este medicamento se aplicó mediante inyección intraperitoneal cada 12 h durante 14 días consecutivos a una dosis por aplicación de 20 mg/kg de peso y suspendida en agua bidestilada libre de pirógenos a 4 mg/ml.

4.2.4. Análisis del crecimiento tumoral. En los estudios de crecimiento de los tumores orto y heterotópicos implantados, de escalamiento de dosis del vector y de eficacia antineoplásica, se determinó el crecimiento de los tumores en los animales en una observación realizada cada 4 días de la siguiente manera. Se medían las longitudes de los diámetros mayor y menor del tumor con un calibrador tipo Vernier. Con estos dos valores se determinaba un diámetro promedio (DP) para cada observación puntual, como se indica en la fórmula 1:

Fórmula 1: (diámetro mayor + diámetro menor)/2= DP

En los estudios de crecimiento de los implantes, se construyó una curva de crecimiento relativo entre los días 8 y 32 post-implantación. El límite inferior de 8 días se debe a que tan sólo en este día había una masa tumoral medible, mientras que el límite superior de 32 días estuvo impuesto por el día máximo en que se conservaron todos los animales vivos para mantener los grupos íntegros durante el experimento. En el caso del estudio de escalamiento de dosis, las curvas se construyeron con los datos proporcionados por estos promedios. En el análisis del crecimiento tumoral de los implantes, los datos recolectados en cada observación ($DP_{día\ x}$) se dividieron por el valor del DP al día de la transducción ($DP_{día\ 0}$) y se multiplicaron por 100 para obtener el índice de crecimiento relativo o ICR (ver fórmula 2). Los valores del ICR de cada tumor en cada punto de la observación se promediaron para cada grupo de implantación y se determinaron los errores estándares.

Fórmula 2: $(DP_{día\ x}/DP_{día0}) \times 100 = ICR$

Para los estudios de eficacia antineoplásica, se agruparon y promediaron los ICR de los animales pertenecientes a los grupos tratados con el vector terapéutico y con el vector control en cada modelo de implantación. A este ICR se le restó 1 para obtener la proporción neta de crecimiento tumoral o PNCT (ver fórmula 3) en cada punto de la observación y se determinaron los promedios y errores estándares para

cada grupo. Con estos datos se construyeron las curvas de crecimiento para analizar la eficacia del tratamiento.

Fórmula 3: $ICR_{día\ X} - 100 = PNCT$

4.2.5. Análisis de sobrevida. En esta fase se trataron grupos experimentales constituidos por 11 ratonas cada uno para el modelo ortotópico y grupos de 10 ratonas cada uno para el modelo heterotópico. Se utilizaron los mismos procedimientos quirúrgicos descritos previamente. También se administró el mismo tratamiento con GCV. Estos animales se dejaron en observación para determinar el máximo periodo de vida después de la administración de los vectores. Aunque algunos fueron encontrados muertos, la mayoría de los animales se sacrificaron cuando el tumor alcanzaba un diámetro mayor de 2.5 cm o cuando el animal presentaba signos de mal estado general como inactividad o lentitud de movimiento, poca reacción a los estímulos, caquexia, enjorobamiento, etc. Con los datos obtenidos se construyó una gráfica de Kaplan-Meier para estudiar los efectos del tratamiento en la sobrevida.

4.2.6. Análisis histopatológico de los tumores. En los estudios de la eficacia antineoplásica, los animales fueron sacrificados mediante dislocación cervical 24 días después de la transducción con el vector adenoviral, debido a que para este día los animales comenzaban a

mostrar signos de mal estado general, principalmente en los grupos controles. Los tumores fueron disecados del cadáver, fijados en formaldehído al 10% y enviados al Departamento de Medicina Comparativa del Baylor College of Medicine para elaboración de laminillas de histología teñidas con hematoxilina y eosina. Las laminillas obtenidas fueron enviadas con tan sólo una identificación de clave al patólogo Dr. Kerby Oberg para la interpretación de los hallazgos en forma ciega, quien además realizó la toma de las microfotografías más relevantes después de haber concluido el análisis de los resultados.

4.2.7. Estudios de linfocitos citotóxicos (LCT) anti-TM40D. Estos estudios se realizaron siguiendo lineamientos previamente descritos (Mule J, 1992). En algunas ratonas utilizadas en los estudios de eficacia antitumoral, se extrajeron los bazos de 5 ratonas de cada grupo de implantación y de tratamiento, inmediatamente después del sacrificio en la campana de flujo laminar en condiciones de estricta antisepsia. Como control no tratado se utilizaron 5 bazos obtenidos de ratonas de la misma edad no implantadas con el tumor y no tratadas con vectores de terapia génica. Como controles positivos se utilizaron 5 machos de la cepa C57BL/6 implantados subcutáneamente con la línea TM40D. Después del sacrificio, los animales fueron rasurados en la región dorsal abdominal izquierda y la piel fue lavada con solución PBS y etanol al 70%. Se utilizaron instrumentos quirúrgicos esterilizados y cajas de Petri con medio

de cultivo RPMI 1640 para la extracción, el depósito y el procesamiento de los bazos obtenidos.

Para el aislamiento de los esplenocitos, los 5 bazos de cada grupo experimental fueron depositados en la misma caja de Petri, lavados con solución de sales balanceada de Hanks (Life Technologies, Gaithersburg, MD, EUA), suspendidos en medio RPMI 1640 y macerados finamente con la superficie plana superior externa del émbolo de una jeringa estéril de 5 cm^3. El macerado fue cuidadosamente depositado en tubos cónicos de 15 ml que contenían Histopaque 1083® (Sigma Diagnostics, St. Louis, MO, EUA) y centrifugados durante 10 min a 800 xg, eliminando el frenado al final del proceso de separación. El paquete linfocitario fue delicadamente recuperado del tubo cónico y se sembró en medio de activación, el cual estaba constituido de medio de cultivo RPMI 1640 enriquecido con 10% de suero bovino fetal (HyClone, Logan, UT, EUA) y 1 U/ml de interleucina murina IL-2 (R&D Systems Inc., Minneapolis, MN, EUA). Antes de la siembra, al final de la resuspensión de las células por pipeteo repetitivo, se tomó una alícuota pequeña para el conteo de las células en un hemocitómetro y se sembraron a una concentración inicial de 2 x 10^6 células/ml. Los linfocitos fueron mantenidos en la incubadora durante 24 h con una atmósfera de 37°C, 5% CO_2 y humedad relativa de 90%. Las células fueron cosechadas y contadas nuevamente, para la realización del estudio de LCT.

El día previo al ensayo también se realizó una resiembra de células TM40D que serían utilizadas como células blanco a una confluencia aproximada del 60-70%. El día del ensayo las células fueron cosechadas, contadas (estimación de un mínimo de 2×10^6 células totales para realizar el ensayo), centrifugadas a 400 xg durante 5 min y resuspendidas en 3 gotas de suero bovino fetal dentro del tubo cónico. El [^{51}Cr] dicromato sódico (ICN Pharmaceuticals Inc, Costa Mesa, CA, EUA) fue resuspendido en medio RPMI 1640 a una concentración de 2.5 µCi/µl. Las células blanco fueron tratadas con 80 µl de [^{51}Cr] dicromato sódico preparado utilizando una jeringa desechable de 1 ml. Las células fueron incubadas durante 90 min utilizando las condiciones de atmósfera mencionadas en el párrafo anterior. Posteriormente las células fueron lavadas dos veces con medio RPMI 1640 suplementado con suero bovino fetal, contabilizadas y resuspendidas a una concentración final de 5×10^4 células/ml. Al final del marcaje, las células efectoras (los linfocitos) y las células blanco (TM40D marcadas) fueron mezcladas en proporciones de 50:1, 25:1, 12.5:1 y 6.25:1 en un formato de 8 x 12 pozos para cultivo celular de fondo redondo. Tres pozos fueron llenados con células TM40D sin linfocitos (cpm espontáneas) y otros 3 pozos fueron llenados con células TM40D tratadas con el detergente Tritón al 1% (Bio-Rad, Hercules, CA, EUA) para referencia del valor de lisis total (cpm máximas). Las células se regresaron a la incubadora con las mismas condiciones de

atmósfera y fueron incubadas durante 4 h. Al final de la incubación, las células fueron centrifugadas a 400 xg durante 5 min y se recuperó el sobrenadante en un segundo plato de formato 8 x 12 de fondo redondo, para la realización de las mediciones de emisión radiactiva en cámara de centelleo y calcular los porcentajes de lisis mediante la fórmula 4 (Mule J, 1992).

Fórmula 4:

(cpm muestra – cpm espontáneas)/(cpm máximas – cpm espontáneas) x 100 = % de citotoxicidad

4.2.8. Análisis estadístico de las curvas de crecimiento tumoral, del escalamiento de dosis, de la eficacia antineoplásica y de sobrevida. Para el estudio del crecimiento natural de los implantes en los dos modelos experimentales, se utilizó la prueba *t* de Student y se realizó un análisis de regresión lineal. En los estudios de escalamiento de dosis se empleó el análisis de varianza tipo ANOVA, mientras que en los estudios de eficacia antineoplásica se utilizó nuevamente la prueba *t* de Student. Para los estudios de sobrevida, se determinó el máximo periodo de sobrevida y el valor de sobrevida 50 (SV_{50}) a partir de la gráfica de Kaplan-Meier; es decir, el día en el que el 50% de los animales en cada grupo de tratamiento aún sobrevivía.

CAPÍTULO V

RESULTADOS

5.1. SUSCEPTIBILIDAD DE LA LÍNEA TUMORAL TM40D AL SISTEMA *HSV-tk*/GCV. Las dos líneas celulares ensayadas mostraron susceptibilidad al vector adenoviral y al sistema *HSV-tk*/GCV. Las dosis letales 50 en cultivo ($TCLD_{50}$) fueron 6.1×10^7 y 2.1×10^7 p.v./ml para la línea TM40D y para la línea control HeLa, respectivamente. Se observó un patrón de comportamiento muy similar entre las dos líneas celulares, que muestra que las células HeLa son más sensible al tratamiento en una ventana de dosis entre 1.2×10^7 y $.1 \times 10^8$ p.v./ml. Después de este punto, las gráficas de toxicidad se entrecruzan, pero esto ocurre a una dosis muy alta (3.3×10^8 p.v./ml), que corresponde aproximadamente a 3.3×10^4 p.v./célula (ver figura 3).

5.2. CRECIMIENTO NATURAL DE LOS TUMORES ORTOTÓPICOS Y HETEROTÓPICOS. La velocidad de crecimiento de los tumores fue constante durante el periodo de tiempo observado, como lo muestra el

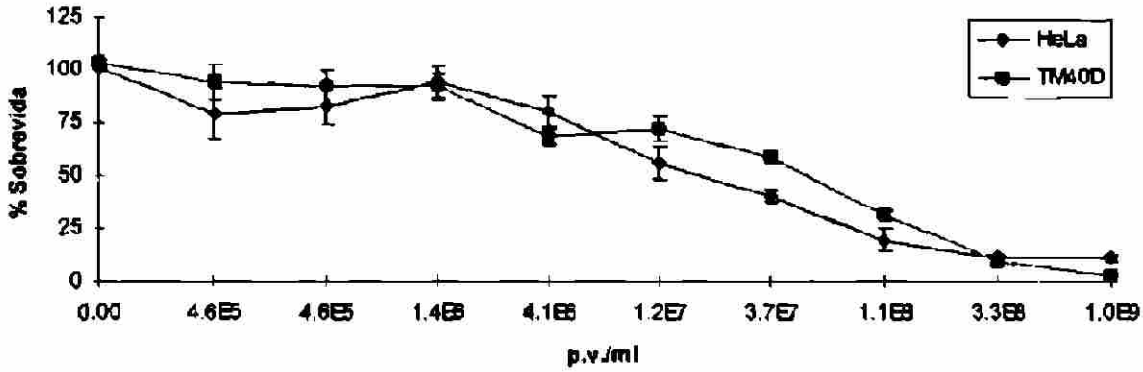

Figura 3. Sobrevida de la línea TM40D tratada con el sistema *HSV-tk*/GCV en condiciones de cultivo de tejidos: Se observan las curvas de sobrevida de las líneas celulares TM40D y HeLa (referencia), tres días después de haber sido tratadas con dosis incrementadas del vector AdV-RSV-*tk* y 10µg/ml de GCV. La sobrevida se estimó a partir de las lecturas de absorbancia de los pozos a 570 nm y comparando éstas con el estándar de absorbancia medido en células de las líneas celulares mencionadas, que no fueron tratadas con el vector, ni con GCV.

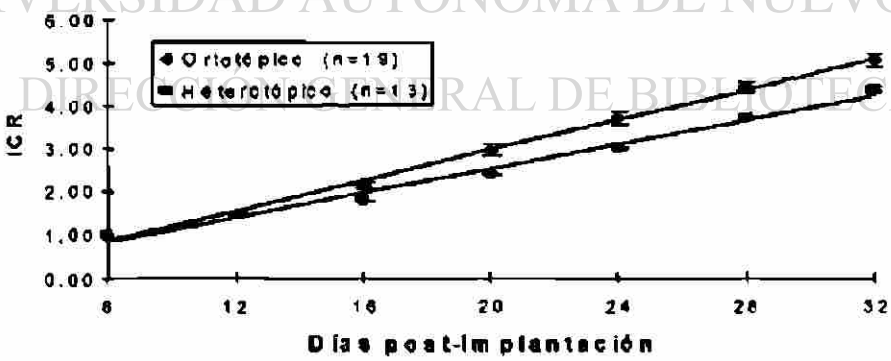

Figura 4. Comparación del crecimiento de los tumores implantados TM40D en los sitios ortotópico y heterotópico: La grafica representa el crecimiento de los tumores a partir del día 8 post-implantación. Los tumores ortotópicos (diamantes), mostraron un crecimiento significativamente más acelerado que los heterotópicos (cuadros).

análisis de regresión lineal en la gráfica correspondiente (ver figura 4). Este análisis permite determinar que los tumores ortotópicos mostraron un tiempo de doblaje de tamaño de 5.9 días, mientras que los tumores subcutáneos mostraron un tiempo de doblaje de 7.6 días. Los tumores ortotópicos crecieron 1.24 veces más rápidamente que los tumores heterotópicos. Al final de la observación (día 24 post-transducción), la diferencia de tamaños entre los tumores de los dos modelos era significativa para un valor de p=0.006.

5.3. ESCALAMIENTO DE DOSIS. La dosis más efectiva para lograr un efecto de inhibición del crecimiento tumoral y libre de efectos tóxicos observables en tumores ortotópicos fue de 3×10^{11} p.v. (1.5×10^{9} p.v./kg de peso) aplicadas intratumoralmente (ver figura 5). Esta dosis mostró diferencia significativa en comparación con las restantes (p=0.05). No se observó inhibición total del crecimiento ni regresión tumoral. Con base en este estudio, se seleccionó la dosis 1×10^{11} p.v. (5×10^{8} p.v./kg de peso) para llevar a cabo los experimentos comparativos de los efectos de la terapia génica en los modelos ortotópico y heterotópico.

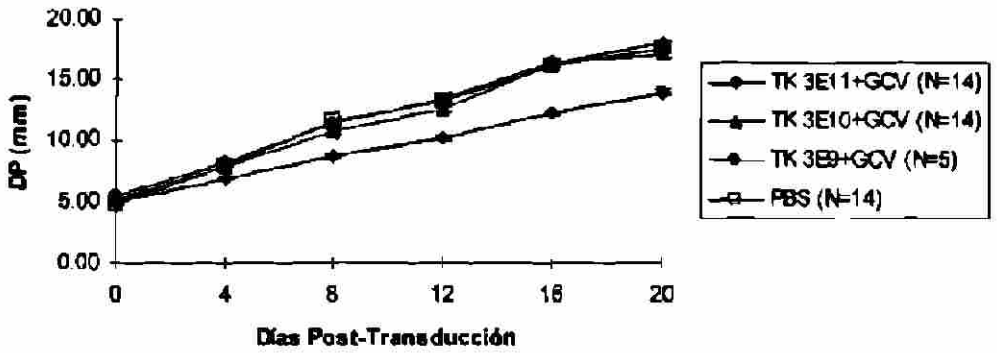

Figura 5. Escalamiento de dosis: Se observan las curvas de crecimiento tumoral en términos de diámetros promedios (DP) de tumores ortotópicos inyectados con dosis incrementadas del vector AdV-RSV-*tk*. Un grupo fue inyectado con PBS como control de no tratamiento. Obsérvese que la dosis de 3.3 x 10^{11} p.v. es significativamente diferente de todas las otras dosis analizadas, las cuales no difieren en efectividad con las del grupo PBS.

5.4. EFICACIA DEL SISTEMA *HSV-tk*/GCV EN LOS MODELOS ORTO Y HETEROPTÓPICO.

5.4.1. Curvas de crecimiento tumoral. Las curvas de crecimiento después de la transducción con el vector, muestran que en el modelo ortotópico hubo una inhibición inicial del crecimiento tumoral que mostró una mayor diferencia entre los grupos en el día 8 post-transducción. Esta diferencia se prolongó hasta el día 16, después del cual, se perdió la diferencia significativa entre los grupos terapéutico y de control (figura 6).

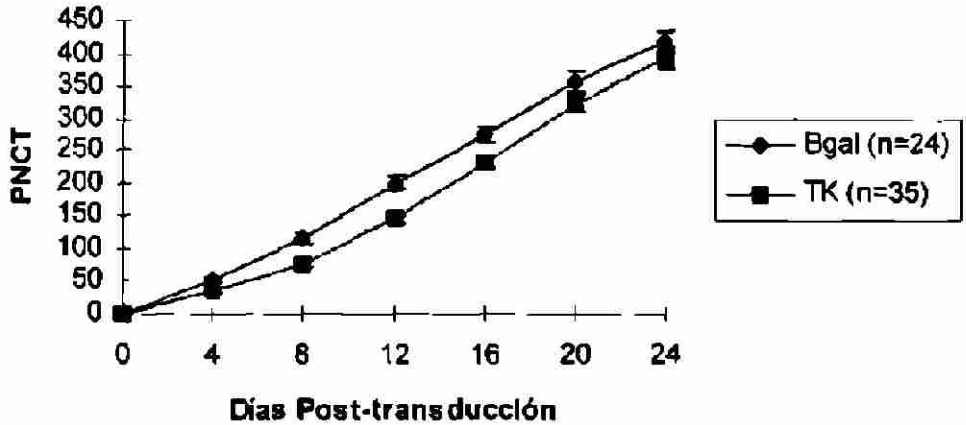

Figura 6. Efecto del sistema *HSV-tk*/GCV en el modelo ortotópico: La supresión del crecimiento tumoral fue evidente y significativamente diferente ($p<0.05$) en el grupo de tratamiento (cuadros) durante los 16 días posteriores a la inyección del vector terapéutico.

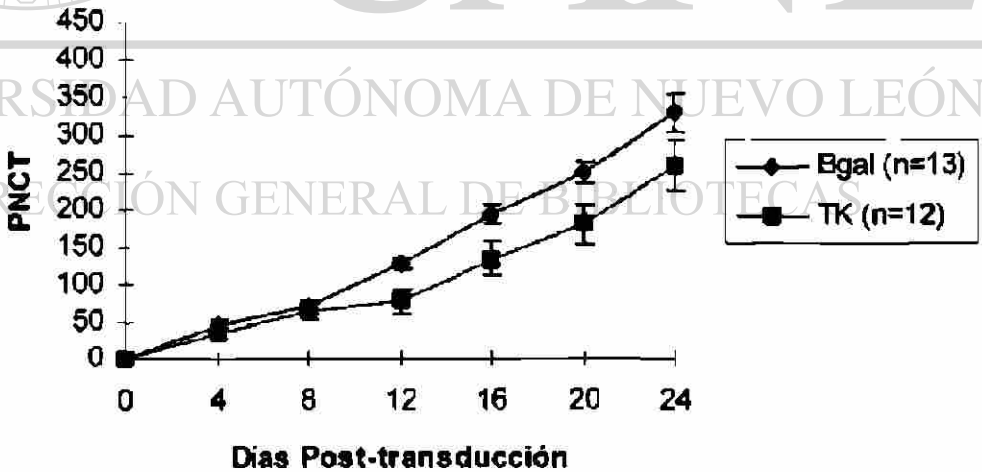

Figura 7. Efecto del sistema *HSV-tk*/GCV en el modelo heterotópico: La supresión del crecimiento tumoral en el grupo terapéutico (cuadros) solo fue evidente después del día 8 post-transducción. Después de este punto, el crecimiento tumoral fue reducido, en comparación con el control ($p<0.05$).

En el modelo heterotópico no se observó un efecto inicial de inhibición tumoral. Este efecto comienza a notarse en el día 12 después de la transducción, día en el cual se observa también la máxima diferencia entre los dos grupos. A partir de entonces, se mantiene una diferencia estadísticamente significativa entre los grupos terapéutico y control, la cual disminuye en el último punto de la observación (ver figura 7).

5.4.2. Análisis histopatológico de los tumores. Los tumores implantados en la glándula mamaria mostraron una celularidad incrementada con respecto a los tumores heterotópicos (ver figuras 8A y 8B). En los tumores ortotópicos (figura 8A) fue frecuente la observación de necrosis focal acompañada de hemorragia, lesiones sugestivas de isquemia focal de un tumor de crecimiento rápido. El tumor viable era denso y carecía de un infiltrado celular inflamatorio significante. Aún en algunos ganglios linfáticos mamarios que ocasionalmente fueron engolfados por la neoplasia, tan sólo se observó una pobre respuesta, evidenciada por la presencia de pocos centros germinales en el ganglio (ver figura 8C). También se observó que estos tumores estimulaban el crecimiento vascular. Vasos de tamaño moderado (50-100 μm de diámetro) fueron observados en los márgenes del tumor y también se observaron numerosos capilares infiltrando la neoplasia (ver figura 8A).

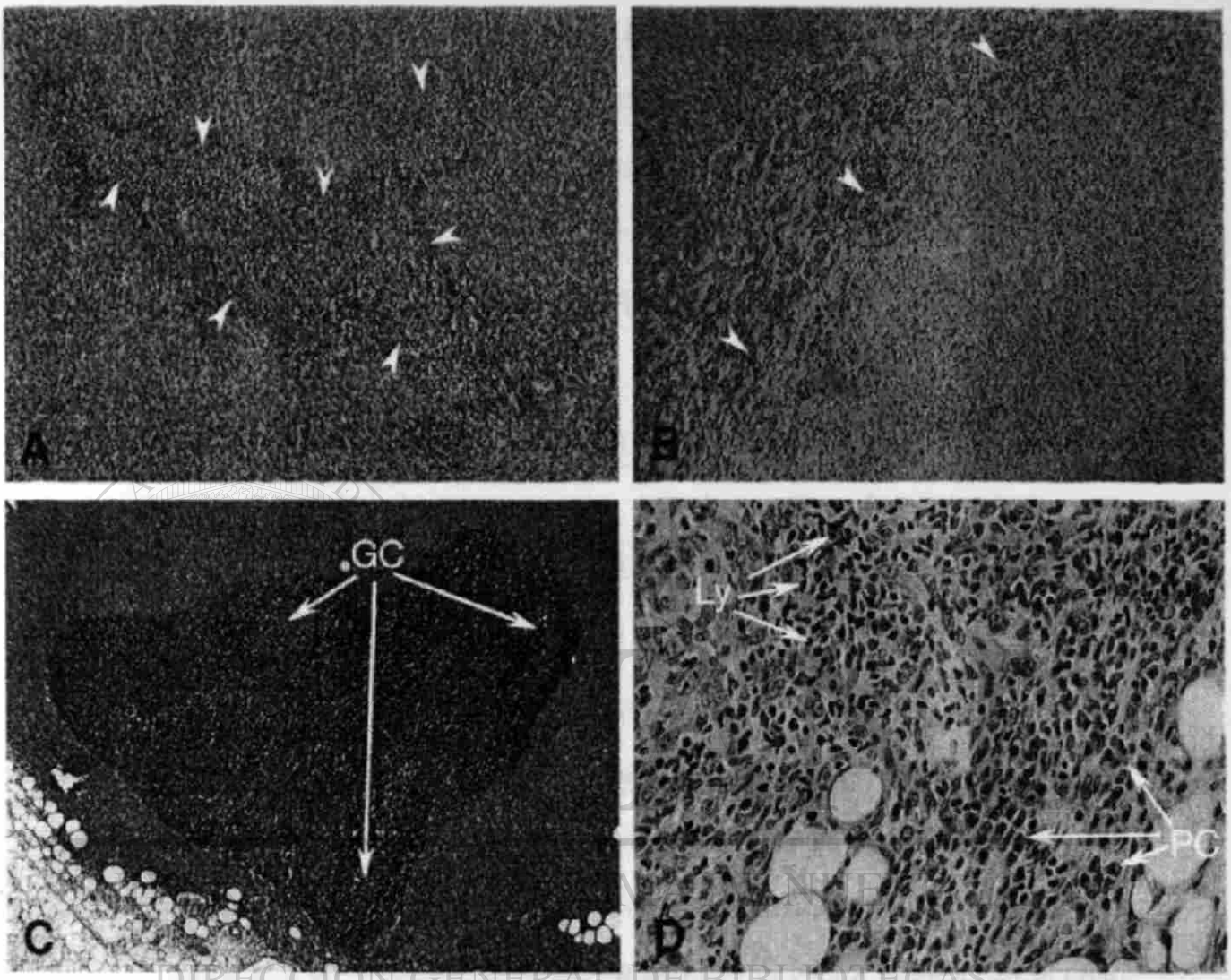

Figura 8. Análisis histopatológico de los tumores ortotópicos y heterotópicos tratados con el sistema *HSV-tk*/GCV: Los tumores ortotópicos muestran lesiones de necrosis focal (A), mientras que los tumores heterotópicos (B) muestran un patrón de necrosis periférica extensa acompañada de infiltración de linfocitos y células plasmáticas (D). Aunque en el modelo ortotópico, algunos nódulos linfoides mamarios fueron invadidos por el crecimiento tumoral (C), la activación de linfocitos no es evidente, como lo demuestra la presencia de escasos centros germinales. A, B y C: Magnificación 60X. D: magnificación 250X. Las flechas blancas señalan los bordes de necrosis. GC: Centros germinales, PC: Células plasmáticas, Ly: Linfocitos.

En contraste, las masas subcutáneas demostraron una necrosis más extensa en la periferia del tumor (ver figura 8B). Numerosos linfocitos infiltrantes y grupos de células plasmáticas estaban presentes en los márgenes viables de la neoplasia. Los tumores heterotópicos eran menos densos y presentaban vasos de tamaño moderado, principalmente en la periferia (ver figuras 8B y 8D).

5.4.3. ANALISIS DE SOBREVIDA. Este estudio no mostró ventajas de sobrevida para los grupos que recibieron el vector terapéutico en ninguno de los dos modelos (ver figura 9). Se observa un mayor tiempo de sobrevida para los animales implantados con los tumores subcutáneos que se prolonga hasta el día 40 post-transducción, en comparación con los animales del modelo ortotópico, cuya sobrevida llega hasta el día 32 post-transducción. Los SV_{50} para el modelo heterotópico fueron 36 días para el grupo tratado con el sistema *HSV-tk*/GCV y 40 días para el grupo tratado con el vector control. En el caso del modelo ortotópico, los SV_{50} fueron de 24 días para ambos grupos experimentales.

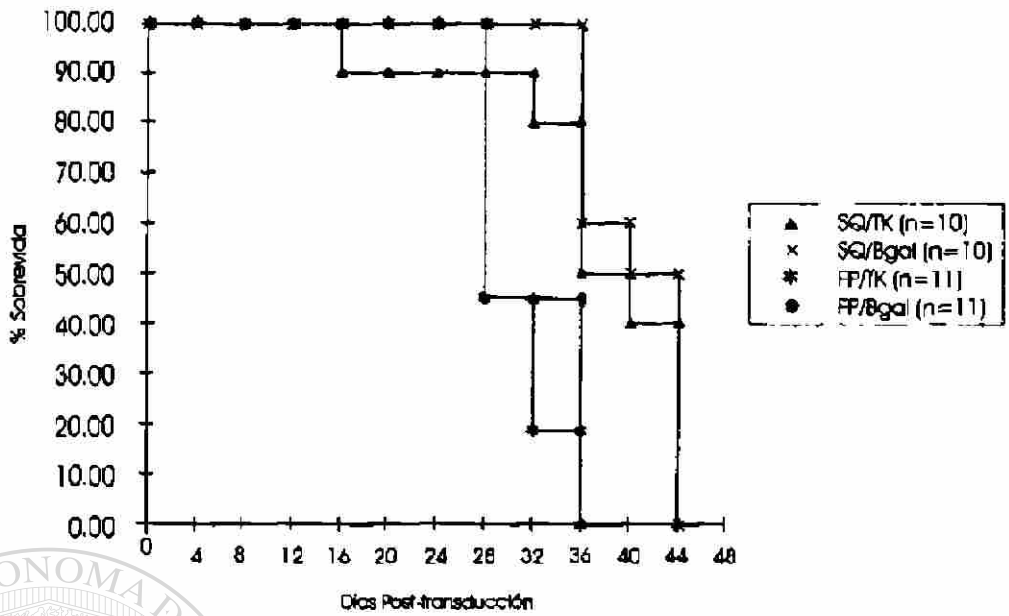

Figura 9. Análisis de sobrevida: Gráfica de Kaplan-Meier, mostrando simultáneamente la sobrevida en los modelos orto y heterotópico. No se observa ningún efecto de incremento de la sobrevida en el grupo terapéutico con respecto al grupo control, en los dos modelos representados en la gráfica.

5.5. PRUEBA DE LINFOCITOS CITOTÓXICOS ANTI-TM40D. Este análisis no demostró la activación de la respuesta celular inmune en contra de la línea tumoral TM40D en los grupos tratados con el sistema *HSV-tk*/GCV en los dos modelos de implantación tumoral (ver figura 10). El estudio muestra que hubo una leve respuesta en los animales utilizados como controles positivos del experimento (ratones C57BL/6), pero el resto de la actividad citolítica permanece cerca de la línea de base en todas las diluciones ensayadas en los distintos grupos.

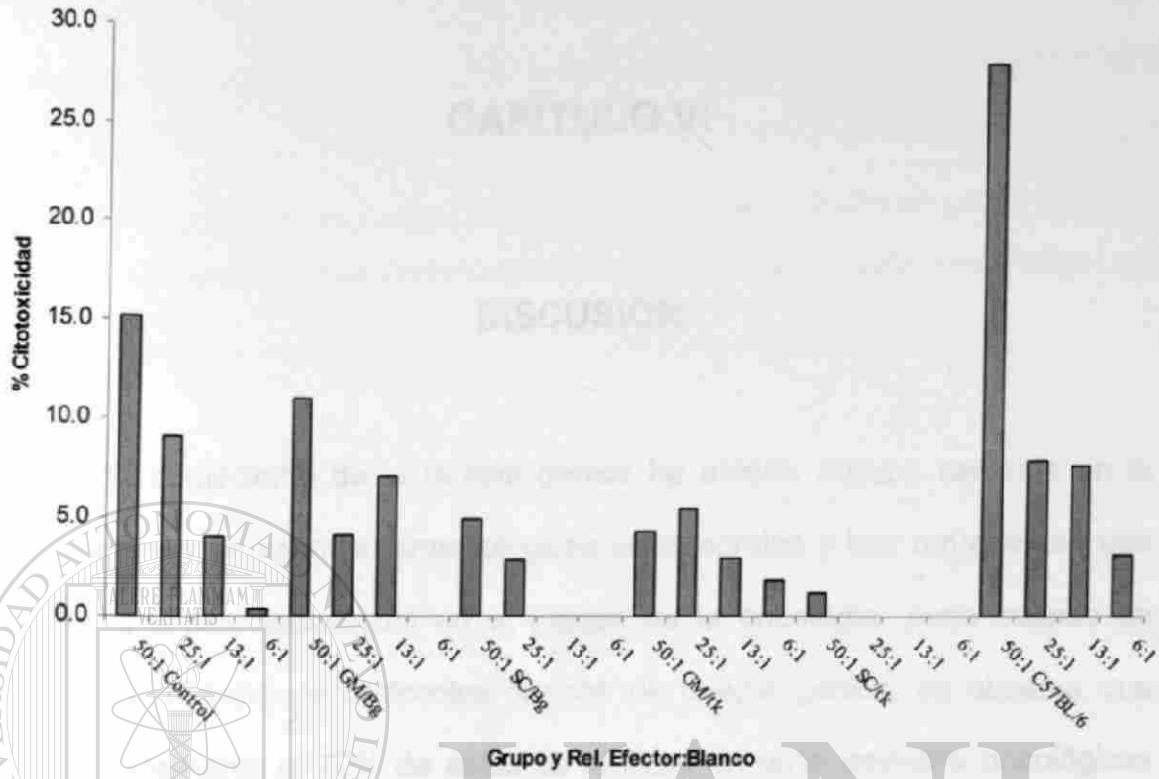

Figura 10. Análisis de linfocitos citotóxicos en los modelos ortotópico y heterotópico tratados con el sistema *HSV-tk*/GCV: La gráfica muestra la actividad linfocítica citotóxica en los dos modelos de implantación, el control no inmunizado (ratonas BALB/c no implantadas) y el control positivo (ratones C57BL/6 implantados con tumor). La única actividad linfocítica relevante se observa en el control positivo en los pozos de relación 50:1 (linfocito:blanco).

CAPÍTULO VI

DISCUSIÓN

El surgimiento de la terapia génica ha abierto nuevos caminos en la investigación de agentes farmacológicos antitumorales y hoy representan una de las grandes esperanzas en el campo de la oncología, pues cuando se analiza el número de protocolos clínicos de terapia génica, se observa que aproximadamente el 70% de estos se realizan en enfermedades oncológicas (Roth y Cristiano 1997). La terapia génica ofrecería algunas ventajas sobre los agentes tradicionales. Por ejemplo, la transducción se puede realizar mediante inyección intratumoral directa (terapia *in-vivo*); las células transducidas se convierten en fábricas de la molécula terapéutica, cuya producción se puede limitar a un tipo celular particular como las células malignas, los linfocitos infiltrantes de tumor o las células endoteliales de la vasculatura tumoral, etc. y el tiempo de expresión puede ser controlado mediante la elección de un vector de vida media corta, el tratamiento *ex-vivo* de un tipo celular particular o mediante la regulación de la expresión génica (Gómez-Navarro y cols. 1999). Otra ventaja es la concentración del producto en el órgano o tejido blanco y el control de la diseminación del agente a los sistemas de circulación sistémica, lo cual evitaría muchos de los efectos colaterales de los agentes

quimioterapéuticos. Sin embargo, la terapia génica se realiza principalmente con vectores virales que son agentes biológicos. Esta naturaleza, determina nuevos e importantes efectos de farmacodinamia y toxicología, algunos de los cuales tienen antecedentes en los virus atenuados utilizados como vacunas, pero en el campo de la oncoterapia, muchos de estos fenómenos están por descubrirse.

Las características terapéuticas de los vectores y genes de terapia génica, como la eficacia, la duración de los efectos, la distribución del agente, la toxicidad y efectos colaterales relacionados, etc, deben probarse, como cualquier otro medicamento, en estudios preclínicos en un modelo animal, generalmente roedores, antes de iniciar protocolos de investigación clínica. El problema fundamental de esta tesis es determinar si el sitio de localización ortotópico o heterotópico de un tumor en un modelo animal, afecta la eficacia de la terapia génica antineoplásica. La observación que el modelo elegido puede afectar la eficacia terapéutica de un agente antitumoral tiene algunos antecedentes en la literatura, principalmente para los agentes quimioterapéuticos, como se discutirá adelante, pero no tiene antecedentes en el área de la terapia génica. La determinación detallada de los factores que producen esta diferencia es por el momento complicada, debido a que aun no existe un cuadro completo de las interacciones entre el tumor y su microambiente y mucho menos, entre tumor, microambiente y agente biológico terapéutico. Sin embargo, en esta tesis se ha realizado un esfuerzo por determinar algunos de estos factores, como la descripción morfológica en el

sitio de tratamiento y la respuesta inmunológica mediada por linfocitos citotóxicos.

Los pasos del desarrollo de esta tesis han sido esencialmente, 1) la selección de un tipo celular maligno que demuestra ser susceptible al tratamiento de terapia génica en condiciones de cultivo de tejidos, 2) la observación del crecimiento natural de los implantes tumorales ortotópicos y heterotópicos en un modelo murino inmunocompetente de cáncer de mama, 3) la determinación de la eficacia antitumoral del sistema *HSV-tk*/GCV mediante la observación de crecimiento tumoral y estudios de histopatología e inmunología y 4) análisis de sobrevida. La discusión de esta tesis se organizó según los resultados obtenidos en cada paso.

Susceptibilidad de la línea celular TM40D al sistema *HSV-tk* mediado por un vector adenoviral. La demostración simultánea de la susceptibilidad de la línea celular neoplásica TM40D al vector adenoviral y al sistema *HSV-tk*/GCV en condiciones de cultivo de tejidos, permite predecir la actividad terapéutica con estos agentes en contra de una masa tumoral de crecimiento rápido en condiciones *in-vivo*, como ocurre en varios ensayos exitosos en modelos animales reportados para esta terapia, como por ejemplo, los ensayos con células C_6 de glioblastoma de rata (Chen y cols. 1994), las células prostáticas malignas RM1 de ratones C57BL/6 (Eastham y cols. 1996), etc.

Comparación del crecimiento tumoral entre los modelos ortotópico y heterotópico. Los tumores del modelo ortotópico crecieron 1.24 veces más rápidamente que los tumores implantados en el espacio subcutáneo. La diferencia en la velocidad de crecimiento puede reflejar el impacto de varios factores. Primero, la glándula mamaria está albergada dentro de un estroma de tejido adiposo. El tejido adiposo cuenta con abundante vascularización que proporciona abundantes nutrientes y óptima oxigenación para favorecer el crecimiento tumoral. Adicionalmente, las células estromales y glandulares sanas pueden proveer factores paracrinos que estimularían el crecimiento neoplásico (Miller y Bukowski, 1994). Los tumores heterotópicos, particularmente en el espacio subcutáneo, frecuentemente producen efectos adversos para el crecimiento tumoral, tal como la formación de una cápsula fibrosa alrededor del tumor (Kyriazis y Kyriaziz, 1980), que pueden inhibir el crecimiento tumoral, acelerar la necrosis y disminuir la actividad metastásica del implante (de Vore y cols., 1980).

Adicionalmente, la temperatura de la glándula mamaria ricamente vascularizada puede ser muy similar a la temperatura corporal del animal, en comparación con la temperatura de los tumores que crecen en el espacio subcutáneo, la cual es potencialmente más baja.

Finalmente, la glándula mamaria ofrece un ambiente con una actividad inmune particular denominada "inmunoprivilegio". Se ha reportado que una baja actividad del sistema de defensa en esta glándula puede favorecer el

crecimiento de tumores en el ratón y en la especie humana (Stewart y Hepner, 1997). El tejido subcutáneo está frecuentemente expuesto al ataque de agentes externos, no es un tejido inmunoprivilegiado y está equipado con abundantes macrófagos tisulares.

Aunque a la fecha no se puede precisar un mecanismo particular, los dos microambientes experimentales difieren en su capacidad de permitir el crecimiento de los implantes realizados con las células neoplásicas TM40D. Algunos reportes previos también han demostrado el crecimiento preferencial de los tumores mamarios en el ambiente ortotópico (Miller, 1981a, Miller y cols., 1981b, Price, 1996), así como el de otros tipos de tumores no mamarios (McLemore y cols., 1988, Hall y Thompson, 1997).

Eficacia del tratamiento con el sistema *HSV-tk*/GCV en los tumores orto y heterotópicos. En los tumores ortotópicos transducidos con el vector *HSV-tk*, se observó una supresión del crecimiento tumoral durante el periodo de tratamiento con GCV y resultó en un máximo de supresión de 33.1% en el día 8 en comparación con los tumores transducidos con el gen *Lac Z*. Poco tiempo después de haberse completado el tratamiento con GCV, los tumores comenzaron a crecer aceleradamente y para el día 20 post-transducción, los tumores alcanzaban el tamaño de los tratados con el vector control y no se observaba un beneficio terapéutico. Los hallazgos histopatológicos en este grupo experimental también reflejaron masas tumorales de crecimiento

expansivo con rica vascularización y áreas de necrosis central, acordes con la observación del crecimiento de la neoplasia.

En contraste, la supresión del crecimiento en los tumores heterotópicos transducidos con el adenovirus *HSV-tk* inicialmente fue menor a la observada en el grupo control. Sin embargo, 6 días después de iniciarse el tratamiento con GCV, el crecimiento de estos tumores fue cercano a un 60% del crecimiento observado en los tumores transducidos con el adenovirus β-gal y este crecimiento fue casi nulo entre los días 8 y 12 en el grupo de tratamiento. Aunque la supresión del crecimiento fue sostenida hasta el día 12, posteriormente las curvas de crecimiento tumoral en los dos grupos se vuelven casi paralelas y de esta manera, se sostiene un efecto significativamente diferente entre los grupos hasta el final del periodo de observación. Los hallazgos histopatológicos mostraron focos de necrosis en la periferia de los tumores tratados e infiltración de células inflamatorias, un patrón no observado en el modelo ortotópico.

La diferencia de eficacia del tratamiento entre los dos modelos era un evento esperado. Sin embargo, se anticipaba que la rica vascularización de los tumores implantados en la glándula mamaria facilitaría el acceso y la distribución del GCV al interior del tumor y que se observaría una mejor respuesta terapéutica. Ya que se observó una respuesta diferente, es factible mencionar que otros mecanismos pudieron afectar la respuesta terapéutica.

Se han propuesto tres mecanismos de acción terapéutica para el sistema *HSV-tk*/GCV que incluyen la inhibición de la división celular por bloqueo de la síntesis de ADN, el efecto testigo y la estimulación del sistema inmune en contra del tumor. Puede haber diferentes explicaciones basadas en estos tres mecanismos. Aunque la vascularización de los tumores heterotópicos puede haber sido más limitada, la encapsulación puede aumentar el efecto testigo debido a un aislamiento y potenciación relativos de la actividad del sistema. La inmunopotenciación pudo haber jugado algún papel, pero los datos de los estudios de linfocitos citotóxicos sugieren que por lo menos esta respuesta no ocurrió en ninguno de los dos modelos experimentales y los análisis histopatológicos revelaron poca actividad de las células del sistema inmune, particularmente en los tumores implantados en la glándula mamaria, en los que ganglios linfáticos invadidos no se observan activados.

Algunos estudios realizados en modelos ortotópicos y heterotópicos para probar la eficacia de agentes quimioterapéuticos han demostrado diferencias dependientes del sitio de implantación. Por ejemplo, se ha reportado una respuesta disminuida de la ciclofosfamida en tumores murinos subcutáneos en comparación con la respuesta de las metástasis pulmonares experimentales (Smith y cols., 1985). También hay un reporte de una respuesta menor con ácido flavono-acético en tumores subcutáneos de adenocarcinoma de colón en comparación con la respuesta observada en animales con ascitis tumoral intraperitoneal (modelo ortotópico) (Bibby y cols., 1989). Adicionalmente, los modelos heterotópicos han sido utilizados también como modelos de

enfermedad metastásica para probar los efectos de la terapia génica (Caruso y cols., 1993, Chen y cols., 1995, Lechanteur y cols., 1997, etc.). Las metástasis tienden a ser más agresivas y responden menos a la terapia que los tumores primarios. Sin embargo, los datos de esta tesis y los de otros autores (de Vore y cols., 1980) demuestran que la agresividad no es un sinónimo de localización heterotópica. Estas observaciones sugieren que la adaptación del tumor o la progresión para iniciar la fase de invasión son más importantes para contribuir a la agresividad del cáncer.

Estudios de supervivencia. Aunque se determinó una diferencia en el crecimiento de los tumores tratados en el modelo heterotópico durante el periodo viable de observación, el análisis de la curva de Kaplan-Meier muestra que la terapia no ofreció ninguna ventaja para la sobrevida del grupo de tratamiento y solo refleja una mayor supervivencia para el modelo heterotópico, probablemente debida a un crecimiento menos acelerado de la masa tumoral. Es posible que el paralelismo de las curvas de crecimiento en el modelo heterotópico se haya perdido después del periodo de observación, como lo sugiere la tendencia de crecimiento en el grupo de tratamiento en los dos últimos puntos de la observación y esto explique la pérdida del beneficio terapéutico en este parámetro.

El desarrollo de esta tesis permite plantear que la elección del modelo tumoral tiene efectos sutiles sobre el análisis final de la eficacia de un agente de terapia génica a nivel observacional. Los resultados obtenidos sugieren que

debe incrementarse la investigación en el área, la cual debería aplicarse a las diferentes estrategias experimentales antineoplásicas en este tipo de terapia, a los diferentes tipos de vectores y a los diferentes tipos de genes terapéuticos. También se sugiere que los estudios preclínicos deben hacer algún énfasis sobre el tipo de modelo a escoger, principalmente en los casos en que la investigación pretenda pasar a la fase clínica.

CAPÍTULO VII

CONCLUSIÓN

El presente trabajo demuestra que hay diferencias de eficacia terapéutica para el sistema *HSV-tk*/GCV mediado por terapia génica en tumores mamarios ortotópicos y heterotópicos generados con la línea celular TM40D. Los análisis histopatológicos revelan tumores de estructura diferente, con patrones de necrosis distintos y la presencia de células inflamatorias en los tumores tratados del modelo heterotópico y poca actividad celular en los centros germinativos de tumores ortotópicos tratados. El ensayo de linfocitos citotóxicos para analizar el efecto inmunológico, no revela diferencias en ninguno de los modelos. La sobrevida tampoco se incrementa en los animales tratados con el sistema *HSV-tk*/GCV en los dos modelos analizados.

Los resultados obtenidos, principalmente en las curvas del análisis del crecimiento de la neoplasia, revelan la importancia de utilizar modelos de implantación tumoral apropiados para examinar la eficacia potencial de las modalidades de terapia génica para el cáncer. Aunque existe un buen número de evidencias sobre la diferencia de efecto con agentes quimioterapéuticos en

diversos modelos de implantación tumoral y metástasis, las observaciones realizadas en este trabajo implican que estas diferencias también pueden observarse con los agentes de terapia génica anticancerosa, pues la eficacia puede depender de varios factores presentes en el microambiente tumoral.

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ANEXO I

PUBLICACIÓN DERIVADA DE ESTA TESIS

Differential Effects of HSV-*tk* Gene Therapy in Orthotopic and Heterotopic Mammary Tumors

(Experimental study)

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Running Title: Site-specific differences of gene therapy

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ABSTRACT

The microenvironment effects on tumor responsiveness to gene therapy were analyzed by comparing TM40D mammary tumor cells implanted into syngeneic mice subcutaneously (heterotopic model) or into the mammary gland (orthotopic model). These sites, although morphologically and embryologically similar, are physiologically different. Animals were separated into 3 groups: 1) a treatment group (HSV-*tk*/GCV), 2) an adenoviral control group and 3) a sham operated group. Tumor growth was measured and tumors histologicallly analyzed to determine the effects of treatment in the two microenvironments. Although both models, resulted in transient tumor growth suppression (33.1%, orthotopic and 39.4%, heterotopic) tumor growth rate and responsiveness to therapy were significantly different. Histologically, the two models displayed different patterns of tumor necrosis, vascularity and immune response. These data indicate that tumor microenvironment may impact the efficacy of gene therapy.

INTRODUCTION

Delivery of genes through viral vectors that generate tumor toxicity is being explored for the treatment of primary and disseminated tumors. Among the strategies for cancer gene therapy, the Herpes simplex virus thymidine kinase gene (HSV-*tk*) followed by ganciclovir (GCV) has been successful in the treatment of tumors in animal models that are refractory to conventional modalities in humans (1, 2). This strategy is based on the conversion of the nucleoside analog GCV into its active intermediate, GCV monophosphate, in cells transfected by HSV-*tk*. The phosphorilated prodrug leads to tumor cell death by halting the synthesis of DNA (3). In addition, untransfected tumor cells can also be affected by exported activated GCV (bystander effect) (4) or through the stimulation of the immune system in response to transfected tumor cells (5, 6). Since therapeutic effectiveness relies on several mechanisms, it may be uniquely susceptible to perturbations in the tumor microenvironment.

Several animal models have been employed to evaluate the effectiveness of HSV-*tk*/GCV therapy. Subcutaneous implantation of prostate (7) and mesothelioma (8) are examples of heterotopic models. Surgical implantation of glioblastoma into brain (9) and chemical (10) or transgenic (11) induction of mammary cancer are examples of orthotopic models. The choice of model utilized is often based on availability and convenience and both heterotopic and orthotopic models have successfully supported the initiation of clinical trials. However, the microenvironment of the preclinical model may significantly affect prognostic value of the clinical outcome.

Among the microenvironmental factors that may influence the therapeutic outcome are stromal architecture, local tissue response, patterns of vascularization, and immune-surveillance. The purpose of this study was to compare the efficacy of the HSV-*tk*+GCV strategy in orthotopic and heterotopic microenvironments using a mammary tumor model.

MATERIALS AND METHODS.

Tumor cells: The mouse mammary tumor cell line TM40D was derived from transplantation of the FSK mammary cell lines into mammary fat pads of syngeneic female BALB/c mice (12). Cells were maintained in α-MEM medium (Life Technologies. Gaithersburg, MD) supplemented with 10% bovine calf serum at 37°C in a 5% CO_2 air atmosphere. Cells for injection into mice were harvested from monolayer cultures by treatment with 0.25% trypsin (Life Technologies. Gaithersburg, MD), washed once and suspended in PBS buffer for injection.

Adenoviral vectors. The AdV-RSV-*tk* contains the HSV-1 thymidine kinase gene (HSV-*tk*) driven by the Rous sarcoma virus (RSV) long terminal repeat in an adenovirus type 5 backbone (2). The control vector (AdV-CMV-β-gal) has the same backbone, but contains the E. coli *lac-Z* gene driven by the human cytomegalovirus immediate-early promoter (HCMVIE). Vectors were produced and quantified in the Baylor College of Medicine Gene Vector Laboratory as described (13).

Orthotopic and Heterotopic tumor implantation. Five to six week old BALB/c females (Harlan Sprague Dawley, Inc. Indianapolis, IN) were used to create the orthotopic and heterotopic tumors. For orthotopic tumors (n=78), mice were placed under general anesthesia with pentobarbital (Nembutal®, Abbott Laboratories. Chicago, IL) at 50 mg/kg. A right abdominal paramedial incision followed by an inguinal incision was performed to expose the left fat pad No. 4 (mammary gland). The mammary gland was injected with 5x 10^4 TM40D cells using an injection volume of 20 µl as described (14). Heterotopic tumors (n=38) were injected subcutaneously in the right flank with 5x 10^4 cells in 50 µl.

Vector delivery and GCV treatment. AdV-RSV-*tk*, AdV-CMV-β-gal or phosphate buffered saline were delivered by intra-tumor injection (20 µl) with an average tumor volume of 40 mm^3. Animals received GCV intraperitoneally at 20 mg/kg b.i.d. for 6 days using an injection volume of 100 µl.

Data analysis. The size of each tumor was calculated every four days as the average of the largest and smallest diameters. The animals were sacrificed when any length reached 2.5 cm or if the animals were visibly ill. The mean diameter of the tumor was compared with its value at the time of vector delivery to determine its relative growth ratio. Student's T-test was used for statistical analyses of treated versus control groups. Tumor density, vascularity, necrosis/apopotosis, and immune response were assessed while blinded to the treatment protocol.

RESULTS/DISCUSSION

Comparison of tumor growth following orthotopic and heterotopic implantation.

Orthotopic tumors grew 1.24 times faster than their heterotopic counterparts. Growth rates for the tumors in both locations were constant over the period studied (Fig. 1 A).

Orthotopic tumors exhibited increased tumor cellularity compared to heterotopic tumors (Figure 2A and B). In orthotopic tumors, focal necrosis accompanied by hemorrhage was observed suggesting focal ischemia, likely from rapid tumor growth (Figure 2A). The surrounding viable tumor was dense and lacked significant inflammatory cell infiltrate. Even though occasional mammary lymph nodes had been engulfed by the expanding tumor, little response was mounted as evidenced by the minimal number of germinal centers present (Figure 2C). This tumors stimulated vascular growth. Moderate sized (50-100 µm) vessels were seen at

the tumor margin (Figure 2C) with numerous capillaries percolating throughout the tumor.

In contrast, subcutaneous tumors demonstrated more extensive necrosis around the tumor periphery. Numerous infiltrating lymphocytes and clusters of plasma cells were present at the viable tumor margin (Figure 2B and D). Furthermore, the tumor was less dense throughout and had fewer moderate sized vessels at its periphery. These data indicate that vascular access, the mechanism of necrosis, and immune response are different at the two locations.

Microenvironment is also likely to impact other parameters that are important for the biologic response to therapy. Even in systemic therapeutic approaches, such as chemotherapy, differences correlated to the site of tumor implantation have been demonstrated. For example, decreased response to cyclophosphamide was found in murine tumors growing in the subcutis in comparison with the response of lung metastases (15). Similarly, treatment of disseminated colon adenocarcinoma was found to be more responsive than subcutaneous implants when treated with flavone acetic acid (16). Gene therapy differs from chemotherapy on the possibility to deliver the therapeutic agent directly into the tumor mass, exerting a more local effect. Considering this difference, the selection of the appropriate tumor model may be even more important for accurate correlation with therapeutic outcomes. Hence, in developing preclinical models for gene therapy it is not only important to consider the cell type, but also the *in-vivo* site.

Efficacy of HSV-tk+GCV therapy on orthotopic and heterotopic tumors.

Treatment of orthotopic tumors with HSV-*tk* and GCV resulted in a significant reduction of tumor growth rate (33.1% at day 8). After completing of the GCV infusion, tumor growth resumed and by day 20 the treated tumor size approximated that of controls. Thus, no long term therapeutic benefit was realized (Fig. 1B). In the heterotopic model, inhibition of tumor growth was not observed until after the GCV delivery was completed (day 8). In this model, maximal

suppression of growth rate (39.4%) was achieved by day 12 and although tumor growth continued, tumor size remained significantly less than controls for the duration of the experiments. Although survival following therapy in the heterotopic model was greater (TD_{50} 39) than in the orthotopic model (TD_{50} 29), neither reached a difference that was significant from their model specific controls (data not shown).

The basis for the difference in therapeutic responsiveness in these two sites is likely related to the mode of therapy. Three mechanisms have been proposed for the action of HSV-*tk*/GCV therapy: 1) direct inhibition of DNA synthesis, 2) bystander effect and 3) immune stimulation. Several parameters of the microenvironment are likely to affect the efficacy of these mechanisms. For example, the site of implantation affected tumor architecture, vascularization and growth rate. In turn, tumor architecture may impact vector distribution and the bystander effect. Vascularization affects drug delivery and host immune response. Growth rate directly affects the DNA synthesis and thus the cytotoxicity of the nucleotide analog. In this report, we demonstrated differences in tumor growth, morphology, immune response and effect of HSV-*tk*/GCV therapy in orthotopic and heterotopic mammary tumors models. Our results highlight the importance of utilizing an appropriate site-specific cancer models to examine the efficacy of gene therapy modalities.

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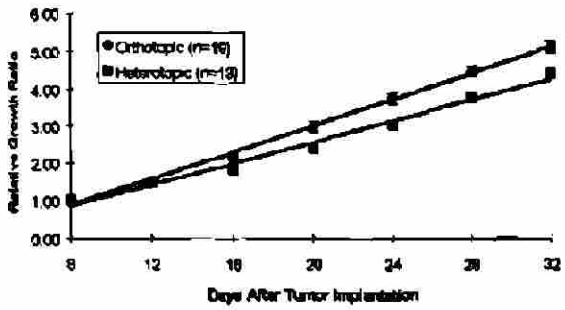
FIGURE LEGENDS:

Figure 1. Relative tumor growth rate in untreated TM40D tumors and during HSV-*tk*/GCV treatment in the orthotopic and heterotopic models. A) Comparison of TM40D tumor growth at orthotopic and heterotopic sites: The graph represents tumor growth seven days after implantation (vector delivery day). Orthotopic tumors (diamonds), showed a significantly faster growth rate than their counterparts (squares). B) Effect of HSV-*tk*/GCV treatment in the orthotopic model: Tumor growth suppression was evident and statistically significant ($p<0.05$) for 16 days after vector delivery. Beyond that time period , the difference between treated and control tumor size was no longer significant. C) Effects of the HSV-*tk*/GCV treatment in the heterotopic model: Tumor growth suppression was not evident until 8 days after treatment.. Thereafter (days 12-24) tumor size is significantly reduced ($p<0.05$) compared to that of the controls.

Figure 2. Comparison of Necrosis, Vascularity and Immune Response in Orthotopic and Heterotopic Tumors.
Focal regions of necrosis were more common in orthotopic tumors (A), while in heterotopic (subcutaneous) locations (B), the tumors displayed more extensive peripheral necrosis accompanied by a lymphocytic and plasma cell infiltrate (D). Although occasional mammary lymph nodes were engulfed by tumor in the orthoptopic model (C), lymphocyte activation was not evident (note the infrequent compact germinal centers). A, B and C magnification 60X, D - 250 X. White arrows indicate the border of necrosis, GC-germinal centers, PC-plasma cells, Ly-lymphocytes.

Figure 1.

A)



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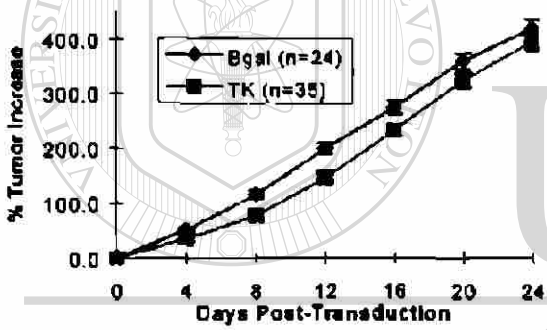

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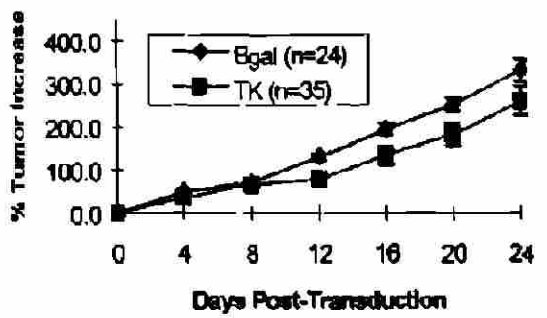
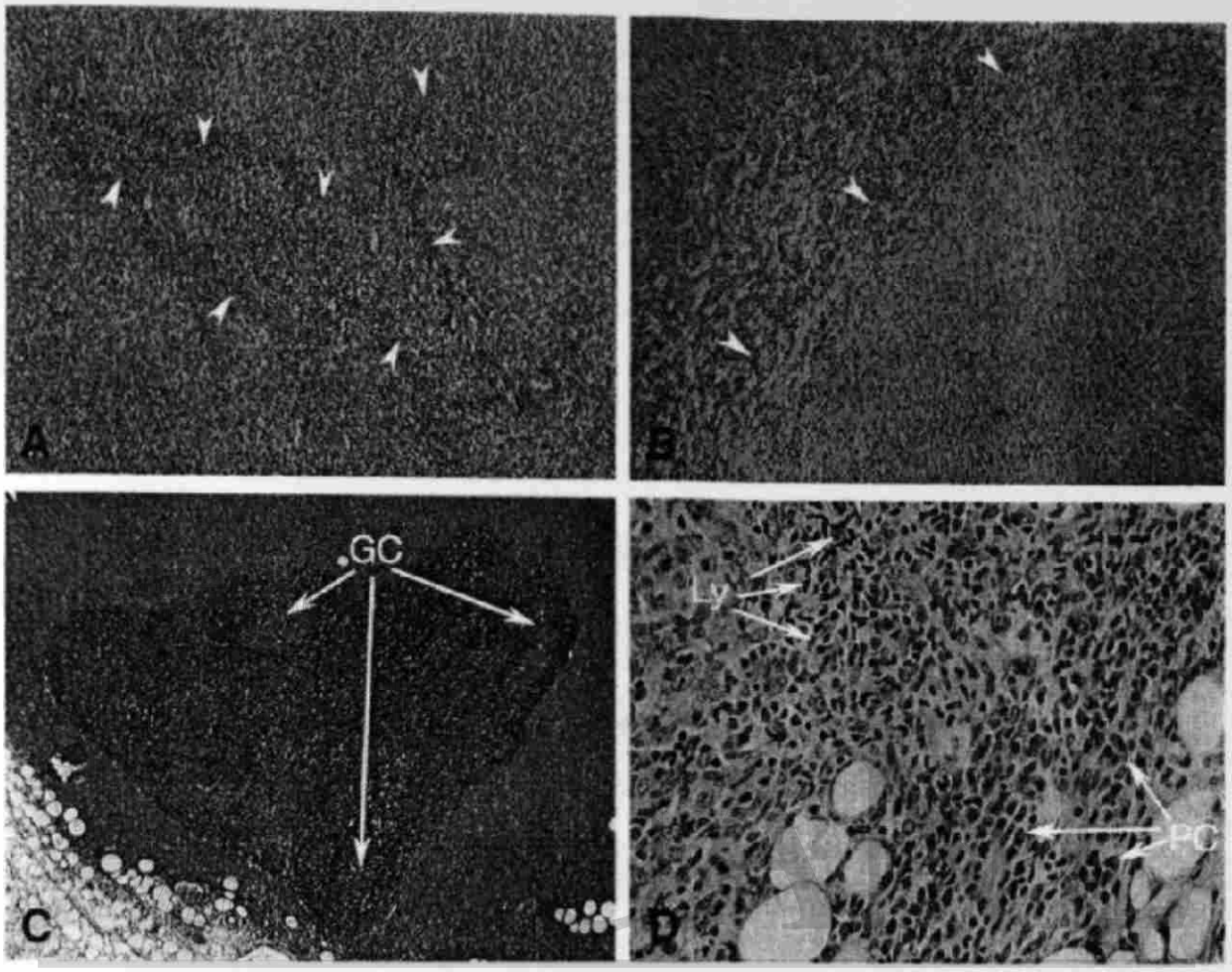


Figure 2.



ANEXO II

PUBLICACIONES ASOCIADAS A ESTA TESIS

Distribution, persistency, toxicity, and lack of replication of an E1A-deficient adenoviral vector after intracardiac delivery in the cotton rat

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Adenoviral vectors were inoculated via intracardiac injection into 5- to 10-week-old cotton rats (*Sigmodon hispidus*) to evaluate the effects of systemic delivery. Cotton rats were chosen as a model because they are semipermissive to the replication of human adenoviruses. The vector used was AdV.RSV-tk, a replication-deficient adenovirus with a *herpes simplex virus thymidine kinase* gene inserted in the E1 region. Vector doses were 3×10^8, 3×10^9, and 3×10^{10} viral particles per animal with and without ganciclovir at 10 mg/kg twice a day. Animals were sacrificed and necropsied at 24 hours, 7 days, and 14 days postinoculation. Gross and microscopic pathologic observations in dosed groups were compared with an unmanipulated control group. From each animal, 10 different organ systems were analyzed for histopathology and vector distribution. The only significant microscopic lesions observed were epicardial inflammation and splenic hemosiderosis. Vector sequences persisted throughout the 14-day assay with preponderance in the heart, lung, and lymphoid organs. Infectious virions were detected for 24 hours, and these virions were only detected at the site of injection of two animals in the highest dose group. No viral replication was detected. Therefore, systemic delivery of up to 3×10^{11} viral particles/kg was well tolerated in this semipermissive host model and did not result in any significant pathology.

Key words: Cotton rat; replication-deficient adenovirus; systemic delivery; herpes simplex virus thymidine kinase.

The increased use of adenoviral vectors in clinical gene therapy highlights the importance of many aspects concerning the interaction of the recombinant vector with the host. Among these, distribution, persistence, pathogenicity, and viral replication are primary concerns that need to be addressed before intentional or incidental systemic inoculation in humans. Since some of the toxicity caused by adenoviral vectors may be a result of residual viral protein expression, analysis in a host which permits the expression of viral proteins may be necessary to fully appreciate potential toxicities. Cotton rats (*Sigmodon hispidus*) provide a unique animal model that can support the replication of human adenoviruses (AdVs) and develop a pathological picture similar to that seen in humans.[1-6] Unfortunately, there is currently only one commercial source of cotton rats in the U.S. (Virion Systems, Rockville, Md); thus, the availability of this model for the assessment of the gene therapy applications of adenoviral vectors has been limited.

Adenoviral vectors for current clinical studies have all been E1A deleted, with backbones based on human AdVs of serotype 2 or 5. For this study, a recombinant of serotype 5 with the *herpes simplex virus thymidine kinase* (*HSV-tk*) gene inserted in the E1A region (AdV.RSV-tk) was selected.[7] Cells that express the *HSV-tk* gene after vector transduction become sensitive to the cytotoxic effect of ganciclovir (GCV).[8] In the absence of GCV, AdV.RSV-tk will have only backbone effects and will represent a more generic version of adenoviral vectors. In addition, this vector was chosen because it has been approved for several human clinical trials Recombinant DNA Advisory Committee, National Institutes of Health #9412-098, #9601-144, #9602-147, and #9602-148) with the potential for inadvertent systemic distribution.

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This study analyzed the distribution, replication, and pathogenicity of AdV.RSV-tk after intracardiac delivery in cotton rats. Despite its common name, the cotton rat belongs to the Cricetidae family and is more closely related to hamsters than rats. These animals do not have easily accessible peripheral vasculature; therefore, intracardiac injection was chosen as a route for systemic distribution in this study. A recent study[5] on the effects of intramuscular (i.m.) and intranasal (i.n.) delivery to cotton rats using 10^8 plaque-forming units (PFUs) per animal detected no vector sequences after 3 and 21 days, respectively. Detection after i.n. delivery was limited to the regional lymph nodes and lungs. The delivery of wild-type AdV i.n. resulted in a peak virus load at 3–5 days postinoculation, with no viral recovery after 14 days. Therefore, it appeared that the duration of adenoviral infections in cotton rats was limited to <14 days, and also that a dose of 10^8 PFUs was well tolerated after i.m. and i.n. delivery. The vector used in these studies had a viral particles (v.p.) to infections unit titer ratio of ≥ 30. Based on those data, a dose of 3×10^8 v.p. was estimated to be a no-effect dose, and 3×10^{10} v.p. was considered a potentially toxic dose for this study. The time points chosen were day 1 postinoculation as the most likely time to find vector sequences, day 7 as the end of GCV treatment, and day 14 as the end of the likely period for finding remaining vector sequences.

MATERIALS AND METHODS

Vector delivery and tissue preparation

The vector used for these studies was AdV.RSV-tk,[7] which was produced at the Baylor College of Medicine Gene Vector Laboratory (Houston, Texas) as previously described.[8] This vector is based on an E1A-deleted backbone of a human AdV type 5, containing a Rous sarcoma virus (RSV) promoter which drives the expression of an *HSV-tk* gene.[10] Infectious titers were analyzed by limiting dilution analysis on the 293 human embryonic kidney cell line.[11] The functionality of the vector lot was tested by *in vitro* transduction of GCV sensitivity to HeLa cells.

A total of 42 5- to 10-week-old cotton rats of either sex were randomly distributed into one control and 13 treatment groups of three animals each. Intracardiac delivery of 100 μL each was performed by percutaneous injection into the upper left quadrant of the thorax using a tuberculin syringe with a 30-gauge needle. Cardiac localization of the needle was determined by blood aspiration prior to vector injection.

Of the total animals, 3 received phosphate-buffered saline, 15 received 3×10^8 v.p. of the vector, 9 received 3×10^9 v.p., and 15 received 3×10^{10} v.p. At 24 hours postinjection, the control group and three animals from each dose group were sacrificed and necropsied. Half of the remaining animals from each dose group received GCV by intraperitoneal injection of 10 mg/kg twice a day for 6 days. At the end of treatment (day 7), three animals from each group were sacrificed and necropsied. One animal at 3×10^9 v.p. and another at 3×10^8 v.p. without GCV died for reasons unrelated to treatment. The remaining animals were sacrificed and necropsied on day 14. At necropsy, samples were taken from blood, gonads, mesenteric nodes, spleen, kidneys, liver, brain, thymus, lungs, and heart, in that order. The order was chosen, starting with those organs least likely to contain vector sequences and continuing through those organs most likely to contain sequences, to minimize the potential for cross-contamination of the samples prior to polymerase chain reaction (PCR) analysis. Part of each tissue was fixed in 10% buffered formalin for histopathologic examination, and a portion was quick-frozen for DNA analysis.

Histopathology

Gross and microscopic observations in dosed groups were compared with those in the control group. Histopathologic assessment was performed in a single-blinded fashion on paraffin-embedded sections of kidney, heart, lung, striated muscle, liver, spleen, thymus, mesenteric lymph node, brain, and gonads that were all stained with hematoxylin-eosin.

DNA analysis

DNA isolation was performed by the overnight digestion of ~100 mg of tissue with 0.88 mg/mL proteinase K in TES buffer (50 mM tris(hydroxymethyl)aminomethane HCl (pH 8.0), 100 mM ethylenediaminetetraacetic acid, and 0.5% sodium dodecyl sulfate (SDS)) at 56°C, followed by treatment with DNAzol reagent according to the manufacturer's instructions (Life Technologies, Gaithersburg, Md). Purified DNA was resuspended in 1 × TES buffer at 25 ng/μL. PCR amplification was performed as previously described.[12]

The primers for PCR amplification were directed toward the RSV-tk/HSV-tk junction of the vector insert sequence (primer RSV 372S: ACGCCATTTGACCATTCACC; primer HSV 244A: CAGTTGCGTGGTGGTGGTTTTC). The identity of the 319-base pair product was confirmed by an internal oligoprobe (AGCGCGTATGGCTTCGTACCCCGGC).

Amplification of the c*K-ras* gene was used as an internal control of DNA quality using primers that have been previously described (primer K1: TATTATAAGGCCTGCT GAAAATGACTGAAT; primer K2: TTACCTCTATTGTT GGATCATATTCGTCCA, product size: 135 base pairs).[13] Primers and probes were purchased from Genosys (The Woodlands, Tex).

Duplex PCR amplification was performed on 150 ng of genomic DNA (~50,000 cell equivalents) in a 15 μL reaction mix containing 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM tris(hydroxymethyl)aminomethane HCl (pH 8.3), 0.33 μM of each primer, 33 μM of each deoxynucleotide triphophate (USB, Cleveland, Ohio), and 1 U of *Taq* polymerase (Fisher Scientific, Pittsburgh, Penn). Forty rounds of amplification were performed in a PTC-100™ machine (MJ Research, Watertown, Mass) with a 94°C denaturation step (30 seconds), a 55°C annealing step (30 seconds), and a 72°C extension (1 minute). Each reaction (7 μL) was electrophoresed in 2% agarose (Bio-Rad Laboratories, Hercules, Calif) and visualized by ethidium bromide staining. Gels were blotted overnight onto 0.2-μm Nytran membranes (Schleicher and Schuell, Keene, NH) with 0.4 N NaOH. Next, 20 pmol of the oligoprobe were 5′ end-labeled in a 50-μL volume reaction with 3 U of T4 polynucleotide kinase (USB) and 12.5 μCi [γ-^{32}P]adenosine triphosphate (Amersham, Arlington Heights, Ill). Prehybridizations were performed at 65°C in 15 mL of hybridization buffer (0.5 M Na_2HPO_4 (pH 7.2), 1 mM ethylenediaminetetraacetic acid, 7% SDS, and 100 μg/mL of fragmented salmon DNA) for 30 minutes. Hybridizations were completed by adding the probe to the prehybridization solution and incubating overnight at 65°C. Filters were washed two times for 20 minutes at room temperature with wash solution (40 mM Na_2HPO_4 and 1% SDS), and autoradiography was completed

Table 1. Vector Sequence Distribution

Day	Dose	GCV	*n*	Blood	Brain	Gonads	Heart	Kidney	Liver	Lungs	Mesenteric nodes	Spleen	Thymus
1	3×10^8	No	3	—	—	—	1	—	—	—	—	1	1
	3×10^9	No	3	1	—	—	3	—	1	3	1	1	3
	3×10^{10}	No	3	—	1	1	2	—	—	3	—	1	2
7	3×10^8	No	3	1	—	1	1	—	—	2	1	2	—
	3×10^8	Yes	3	2	—	—	—	—	—	1	2	1	—
	3×10^9	No	2	—	—	—	2	—	—	2	—	1	1
	3×10^9	Yes	3	—	—	—	3	—	1	3	—	1	3
	3×10^{10}	No	3	2	—	—	3	—	2	2	—	2	2
	3×10^{10}	Yes	3	—	—	—	3	—	1	3	2	1	3
14	3×10^8	No	2	—	—	—	—	1	—	1	1	—	—
	3×10^8	Yes	3	—	—	1	—	—	1	3	2	—	3
	3×10^{10}	No	3	—	—	—	2	1	1	3	1	2	2
	3×10^{10}	Yes	3	—	—	—	3	—	1	3	1	1	2

Numbers indicate the number of tissue samples that were positive from the number of samples tested (*n*).

overnight on X-Omat AR films (Kodak, Rochester, NY) at −70°C.[14]

Plaque assays

Sections of frozen tissues that were found positive for vector sequences by PCR were thawed, ground, and suspended in 1 mL of phosphate-buffered saline. One-tenth of the suspension (100 μL) was plaqued on a confluent monolayer of 293 human embryonic kidney cells in a 24-well plate. These cells are stably transfected with the adenoviral E1 region; thus, they are able to form plaques from E1-deleted vectors and wild-type virus.[9] The detection limit varied according to the organ tested and ranged from 100 to 1000 PFUs/organ (0.1 × fraction of the organ tested).

RESULTS

Vector distribution and persistence

Distribution results are summarized in Table 1. The distribution of the vector was assessed by PCR amplification of the junction sequence of the RSV promoter and the *HSV-tk* gene followed by hybridization using an end-labeled oligonucleotide probe for the HSV-tk region. The sensitivity of this methodology was found to be 1–10 vector particles in the context of 150 ng of cotton rat cellular DNA (Fig 1).

The vector was most often detected in thoracic organs (Table 1; lungs, 29 of 37; heart, 23 of 37; and thymus, 22 of 37) followed by other lymphoid organs (spleen, 14 of 37; and mesenteric lymph nodes, 11 of 37), liver (8 of 37), blood (6 of 37), gonads (3 of 37), kidney (2 of 37), and brain (1 of 37). There was no significant difference in vector distribution correlated with GCV treatment (51 of 150 tissue samples versus 42 of 130 tissue samples, with and without GCV, respectively). The majority of positive blood samples (5 of 6) were found at day 7, with no positives detected at day 14. No other time-correlated differences were observed. Of the tissues from animals that received 3×10^8 v.p., 21% (30 of 140) were positive for vector sequences compared with 38% (30 of 80) and 39% (59 of 150) of tissues from 3×10^9 and 3×10^{10} v.p., respectively.

Gross and histopathology

No gross lesions were observed at the time of necropsy. Histopathologic findings are summarized in Table 2 (only organs with treatment-related abnormalities or a high incidence of background lesions are included).

Treatment-related lesions were observed in the epicardium of the heart and in the spleen. The epicardium was thickened and contained focal to diffuse infiltrations of plasma cells, lymphocytes, macrophages, and occasional polymorphonuclear leukocytes (Fig 2). Epicarditis was limited to the top two vector doses. No epicardial lesions were observed at 3×10^8 v.p. In the spleen, an increased number of hemosiderin-laden macrophages was observed in the red pulp of treated animals at 7 days, but this number returned to normal levels at day 14. A slight difference in these lesions was observed between the lower dose groups without GCV (0 of 5) and those with GCV (4 of 6). The effects of GCV were not apparent at the 3×10^{10} v.p. dose, where both groups had an equal incidence of the lesions.

Background microscopic lesions were observed in the kidney and heart of control and experimental animals.

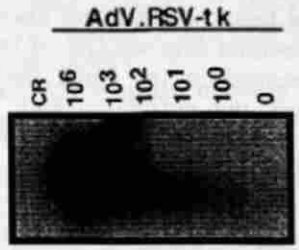

Figure 1. Vector detection sensitivity assay. DNA was extracted from a control cotton rat and quantified by ultraviolet adsorption at 260 nm. AdV.RSV-tk particle concentration was determined by ultraviolet adsorption using a conversion factor of 1.1×10^{12} particles per optical density of a 1:20 dilution. The PCR reactions were all performed in the context of 150 ng of cotton rat DNA and were analyzed by Southern blot. CR, cotton rat DNA alone; 10^6 to 10^0, the calculated number of vector particles in the context of cotton rat DNA; 0, no DNA or vector.

Table 2. Histopathology Results of Adenoviral Vector Toxicity Study in Cotton Rats

	24-Hour necropsy			
	Control	3×10^8 AdV	3×10^9 AdV	3×10^{10} AdV
	Inc/Avg Sev	Inc/Avg Sev	Inc/Avg Sev	Inc/Avg Sev
Lesions				
Kidney				
Nephropathy	3/3 (1.3)	3/3 (1.0)	3/3 (1.7)	2/3 (1.0)
Hemosiderin pigment, tubules				
Heart				
Degenerative cardiomyopathy	2/3 (1.5)	2/3 (2.0)	3/3 (1.7)	1/3 (1.0)
Chronic inflammation, epicardium	0/3	0/3	0/3	1/3 (2.0)
Acute inflammation, epicardium				
Acute inflammation, myocardium	0/3	0/3	1/3 (3.0)	0/3
Spleen				
Acute inflammation, red pulp				
Hemosiderin pigment, red pulp				

The renal lesions consisted of tubular degeneration, tubular regeneration, tubular dilatation, protein casts within tubular lumina, mineralization, and infiltration of mononuclear cells in the interstitium. This nephropathy was similar to that described previously in the rat and mouse.[15,16] The incidence and degree of severity for nephropathy was similar for all experimental and control groups; thus, it was not deemed to be treatment-related. Spontaneous lesions in the heart were limited to the myocardium and consisted of myofiber degeneration, mineralization, and infiltration of macrophages and lymphocytes. This degenerative cardiomyopathy was similar to that described with vitamin E deficiency[17] and has been observed before in the cotton rat colony at our institution (data not shown). The cotton rat appears to be more sensitive to vitamin E deficiency than mice and rats.[18] Again, there was no significant difference in the incidence or degree of severity of this lesion between experimental and control groups. Consequently, it was determined that this lesion was not associated with the treatment. Occasional degenerative or inflammatory lesions were observed in the lung, liver, spleen, and thymus but were not correlated with treatment. No microscopic lesions were observed in striated muscle, lymph nodes, gonads, or brain.

Assays for infectious adenoviral vectors

All of the tissues that were found positive by PCR were analyzed for PFUs to detect the persistence of infectious particles or replication-competent recombinants. PFUs were only detected in samples from the site of injection of two animals at the highest dose, and were only seen at the first time point. In the two PFU-positive animals, 40 and 120 PFUs were detected from undiluted samples.

DISCUSSION

In this study, the distribution and toxicity after the systemic delivery of adenoviral vectors were analyzed in a semipermissive host. The only significant microscopic lesions observed in a time-course analysis of three vector doses (3×10^8, 3×10^9, and 3×10^{10} v.p. per animal) were in the epicardium of the heart and in the spleen. The inflammatory changes in the outer portion of the heart appear to be related to the vector and may be specific to the intracardiac route of administration. Other routes of administration may not have the same effect. Splenic hemosiderosis may have resulted from a hemolytic crisis, visceral hemorrhage, or a defect in iron metabolism. Since this lesion decreased by day 14 of the experiment, the most likely explanation is that the vector caused minimal hemolysis, and that the resulting hemosiderin was cleared by the macrophage system within a week. Thus, systemic delivery of up to 3×10^{11} v.p./kg of AdV.RSV-tk was not significantly toxic in this animal model, even with concomitant administration of GCV.

In contrast to high liver presence after tail vein injections in mice and other animal models, vector sequences were primarily detected in thoracic organs near the site of injection in this study. Although this was surprising, a similar result as observed in another report of systemic delivery to cotton rats.[6] In that study, vectors were delivered i.m. or intravenously. Even though expression from the vectors was found to be high in the liver, v.p. were mostly detected in lung and spleen tissue. The implication of this result is that there may be a differential between vector DNA presence and the level of expression for the vector. Therefore, distribution may be influenced by whether it is associated with the vector product or vector particles. In another report, Yei et al[19]

Table 2. (*Continued*)

7-Day necropsy						14-Day necropsy			
3×10^8 AdV w/o GCV Inc/Avg Sev	3×10^9 AdV w/o GCV Inc/Avg Sev	3×10^{10} AdV w/o GCV Inc/Avg Sev	3×10^8 AdV w/GCV Inc/Avg Sev	3×10^9 AdV w/GCV Inc/Avg Sev	3×10^{10} AdV w/GCV Inc/Avg Sev	3×10^8 AdV w/o GCV Inc/Avg Sev	3×10^{10} AdV w/o GCV Inc/Avg Sev	3×10^8 AdV w/GCV Inc/Avg Sev	3×10^{10} AdV w/GCV Inc/Avg Sev
2/3 (1.0)	2/2 (1.5)	3/3 (1.3)	3/3 (2.0) 2/3 (1.5)	3/3 (2.0) 0/3	2/3 (1.0) 0/3	1/2 (1.0) 0/2	3/3 (1.7) 1/3 (1.0)	3/3 (1.3) 1/3 (2.0)	3/3 (1.3) 1/3 (1.0)
1/3 (1.0) 0/3	2/2 (1.5) 1/2 (2.0)	3/3 (1.3) 2/3 (3.0)	2/3 (1.0) 0/3	2/3 (1.0) 3/3 (2.7)	3/3 (1.7) 3/3 (2.0)	1/2 (1.0) 0/2 0/2	3/3 (2.0) 1/3 (2.0) 0/3	3/3 (1.3) 0/3 0/3	2/3 (1.5) 2/3 (1.5) 1/3 (2.0)
0/3	0/2	3/3 (2.0)	2/3 (2.0)	2/3 (2.5)	2/3 (2.0)	0/2 0/2	0/3 0/3	1/3 (4.0) 0/3	0/3 1/3 (2.0)

Inc = Incidence of lesion.
Avg Sev = Average degree of severity of lesion.
Severity code (in parentheses): 0 = no lesion; 1 = minimal; 2 = mild; 3 = moderate; 4 = marked.

delivered adenoviral vectors to cotton rats via an i.n. route. In that study, the vector was only detected in the lung; it was not detected in any other organs, including liver. The results from those two reports and the distribution pattern observed here may be characteristic of the cotton rat model. Another hypothesis is that the distribution observed may reflect a rapid adsorption of the vector near the site of injection, especially in the lungs, leading to minimal distribution to the liver. Alternatively, the distribution observed may reflect poor systemic dissemination by intracardiac delivery, perhaps due to cardiac muscle uptake. It is unlikely that the latter possibility is the sole explanation, since the second most prominent site for vector sequences was lymphoid organs; in addition, there was an apparent peak of vector presence in the blood at day 7, suggesting some systemic distribution. Since primary vector distribution may be limited by vector sequestration from cells that are proximal to the site of injection, selection of the site of injection may be critical to the design of clinical protocols. A comparative analysis of delivery sites will be required to address this question.

Surprisingly, there were no discernible differences in the presence and persistence of the vector between animals with or without GCV. This may reflect the preponderance of quiescent cells *in vivo* and the relatively innocuous effect of HSV-tk and GCV treatment on nondividing cells. The only exception to this lack of apparent GCV effect was seen at day 7 in the two lower dose groups. Splenic hemosiderosis was not observed in any of the animals without GCV, while 4 of 6 animals with GCV had these lesions. This difference was no longer evident at day 14, possibly reflecting a temporal toxic effect on hematologic tissues that is mostly resolved by day 14. However, the data suggest that at the dose levels tested here, the tk/GCV treatment is not toxic to normal tissues.

Vector sequences were detected in various organs for the duration of the study. No significant correlation was observed between the vector dose used or GCV treatment and the distribution or incidence of detection. This lack of correlation may reflect a stochastic distribution within the organs treated as well as the limited proportion of each organ represented by the DNA used for PCR analysis. However, this possibility would equally affect the various organs and consequently is unlikely to have an impact on the comparative results of the study. Infectious particles were only detected from two of nine animals and were only seen at the site of injection at the first time point (24 hours postinjection). The two animals from which PFUs were detected were from the highest dose group. The absence of PFUs in the majority of animals and at all later time points suggests that the injected infectious virions were not maintained, and that there was no vector replication in the *in vivo* environment. Thus, it is unlikely that infectious vector shedding occurs after systemic *in vivo* transduction with replication-defective adenoviral vectors.

In summary, intracardiac delivery of an E1-deleted adenoviral vector at 3×10^9 to 3×10^{11} v.p./kg was well tolerated in a semipermissive host. There was a dose- and time-related inflammatory response at the site of injection and dose- and GCV-associated splenic hemosiderosis, but this hemosiderosis was resolved by day 14. No major pathology or viral replication was observed in any of the animals.

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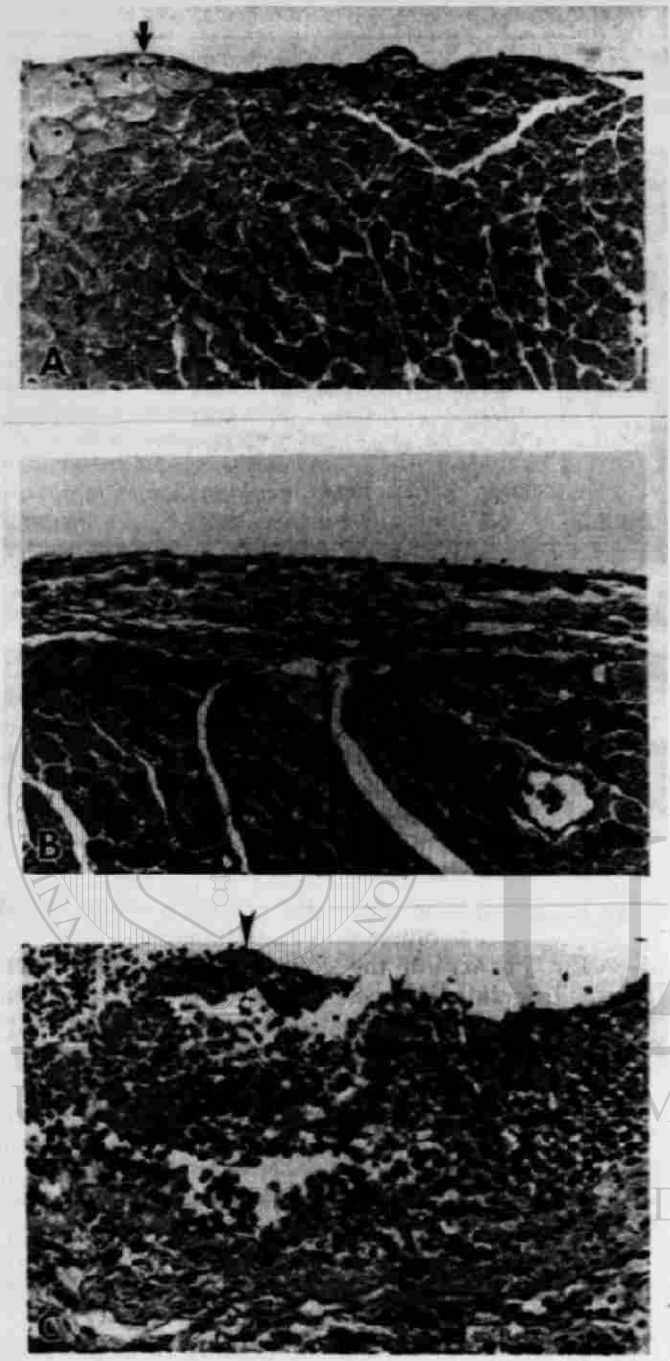

Figure 2. Treatment-related cardiac lesions (×200). **A** shows the normal myocardium (✲) and epicardium (arrow) from a control cotton rat. **B** indicates moderate thickening of the epicardium with diffuse infiltration of lymphocytes and macrophages. **C** shows diffuse thickening of the epicardium with infiltration of lymphocytes, macrophages, and fibrin deposition (arrowheads). **B** and **C** received 3×10^{10} AdV.RSV-tk without GCV and were necropsied 7 days postinoculation.

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In Situ Gene Therapy for Adenocarcinoma of the Prostate: A Phase I Clinical Trial

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ABSTRACT

For patients with local recurrence of prostate cancer after definitive irradiation therapy there is no treatment widely considered safe and effective. After extensive preclinical testing of prodrug gene therapy *in vitro* and *in vivo*, we conducted a phase I dose escalation clinical trial of intraprostatic injection of a replication-deficient adenovirus (ADV) containing the herpes simplex virus thymidine kinase gene (HSV-*tk*) injected directly into the prostate, followed by intravenous administration of the prodrug ganciclovir (GCV). Our goal was to determine safe dose levels of the vector for future trials of efficacy. Patients with a rising serum prostate-specific antigen (PSA) level and biopsy confirmation of local recurrence of prostate cancer without evidence of metastases one or more years after definitive irradiation therapy were eligible for the trial. After giving informed consent, patients received injections of increasing concentrations of ADV/HSA-*tk* in 1 ml into the prostate under ultrasound guidance. Ganciclovir was then given intravenously for 14 days (5 mg/kg every 12 hr). Patients were monitored closely for evidence of toxicity and for response to therapy. Eighteen patients were treated at 4 escalating doses: group 1 ($n = 4$) received 1×10^8 infectious units (IU); group 2 ($n = 5$) received 1×10^9 IU; group 3 ($n = 4$) received 1×10^{10} IU; group 4 ($n = 5$) received 1×10^{11} IU. Vector was detected by PCR of urine samples after treatment, increasing in frequency and duration (up to 32 days) as the dose increased. All cultures of blood and urine specimens were negative for growth of adenovirus. Minimal toxicity (grade 1–2) was encountered in four patients. One patient at the highest dose level developed spontaneously reversible grade 4 thrombocytopenia and grade 3 hepatotoxicity. Three patients achieved an objective response, one each at the three highest dose levels, documented by a fall in serum PSA levels by 50% or more, sustained for 6 weeks to 1 year. This study is the first to demonstrate the safety of ADV/HSV-*tk* plus GCV gene therapy in human prostate cancer and the first to demonstrate anticancer activity of gene therapy in patients with prostate cancer. Further trials are underway to identify the optimal distribution of vector within the prostate and to explore the safety of repeat courses of gene therapy.

OVERVIEW SUMMARY

We conducted a phase I dose escalation clinical trial of intraprostatic injection of a replication-deficient adenovirus (ADV) containing the herpes simplex virus thymidine kinase gene (HSV-*tk*) injected directly into the prostate, followed by intravenous administration of the prodrug ganciclovir (GCV). Eighteen patients were treated at 4 escalating doses from 1×10^8 to 1×10^{11}. No or minimal toxicity (grade 1–2) was encountered in most patients. One patient

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at the highest dose level developed spontaneously reversible grade 4 thrombocytopenia and grade 3 hepatotoxicity. Three patients achieved an objective response, one each at the three highest dose levels, documented by a fall in serum PSA levels by 50% or more sustained for 6 weeks to 1 year. This study is the first to demonstrate the safety of ADV/HSV-*tk* plus GCV gene therapy in human prostate cancer and the first to demonstrate anticancer activity of gene therapy for patients with prostate cancer.

INTRODUCTION

EARLY STAGE PROSTATE CANCER, localized within the prostate, may be managed conservatively or treated actively with radical prostatectomy or irradiation therapy. Of the 184,500 new cases expected this year, more than 80% will be early stage and a third of these will be treated with definitive irradiation therapy (Landis *et al.*, 1998). While modern radiotherapy usually controls the local tumor, some patients will develop local recurrence within the prostate that becomes evident through a rising serum prostate-specific antigen (PSA) level and a positive needle biopsy result in the absence of evidence of metastases. For the estimated 10,000–15,000 patients with local recurrence each year, there is no accepted, standard treatment. Androgen ablation, whether early or delayed, is palliative and radical prostatectomy, although sometimes curative, carries substantial risks (Rogers *et al.*, 1995). Transfer or insertion of genes that (1) result in the activation of prodrugs to produce cytotoxicity (Moolten, 1986; Moolten and Wells, 1990), (2) replace inactivated tumor suppressor genes (Bacchietti and Graham, 1993; Wills *et al.*, 1994; Zhang *et al.*, 1994), or (3) stimulate the immune system (Vieweg *et al.*, 1994), offers new, potentially effective therapeutic approaches to the treatment of prostate cancer. One method of cytotoxic gene therapy is viral transduction of the herpes simplex virus thymidine kinase (HSV-*tk*) gene followed by systemic administration of the nucleoside analog ganciclovir (GCV). The HSV-*tk* gene product phosphorylates GCV, which is incorporated into DNA during cell division and leads to cell death. Moreover, the number of cells killed significantly exceeds the number of cells transduced with the HSV-*tk* gene (Freeman *et al.*, 1992). This phenomenon, the "bystander effect," may be caused by the passage of toxic metabolites across gap junctions, the uptake of the products of apoptotic bodies, a local immune response, and/or inhibition of endothelial cells (Freeman *et al.*, 1993; Vile *et al.*, 1994; Elshami *et al.*, 1996; Hamel *et al.*, 1996; Ramesh *et al.*, 1996a,b). Adenoviral HSV-*tk*/GCV gene therapy has been reported in clinical trials of patients with brain tumors (Alavi *et al.*, 1998), mesotheliomas (Sterman *et al.*, 1998), and ovarian carcinomas (Alvarez and Curiel, 1997).

We demonstrated significant cytotoxicity induced by HSV-*tk*/GCV in both mouse and human prostate cancer cells *in vitro* and in mouse prostate carcinomas *in vivo* (Eastham *et al.*, 1996). HSV-*tk*/GCV treatment-induced cytotoxicity *in vivo* was accompanied by increased areas of necrosis, increased numbers of apoptotic bodies, and extensive immunocyte infiltration relative to controls. In addition, we produced significant growth inhibition of local prostate cancer together with suppression of spontaneous and induced metastases, in an orthotopic *in vivo* mouse model of prostate cancer when the vector was injected directly (*in situ*) into local tumor within the prostate gland (Hall *et al.*, 1997). Further, we have reported no systemic spread and minimal local spread of the adenoviral vector within the genitourinary system in our mouse model (Timme *et al.*, 1998). To determine the efficacy of this form of suicide gene therapy against human prostate cancer, we initiated a phase I dose escalation trial of a replication-deficient adenoviral vector carrying the HSV-*tk* gene followed by the intravenous administration of GCV in men with local recurrence of prostate cancer after irradiation therapy (IND 6636). Herein, we report the results of this trial.

MATERIALS AND METHODS

Treatment protocol

The protocol was approved by the Baylor Institutional Review Board (IRB) (H-3826) on April 3, 1995 and by the Food and Drug Administration (BB-IND 6636) on May 1, 1996. Patients reviewed the informed consent document and were individually counseled before agreeing to participate in this study. Counseling included a thorough discussion of alternative treatments including watchful waiting, salvage radical prostatectomy, cryosurgery, and androgen ablation.

Eligibility criteria

Patients were eligible for this phase I clinical trial if they had evidence of local recurrence of cancer within the prostate, proven by biopsy, at least 1 year after completion of irradiation therapy. They must have had evidence of a biologically active tumor with a rising serum PSA level on at least three occasions at least 2 weeks apart. Patients must have had no evidence of metastases (a normal serum acid phosphatase, pelvic and abdominal computerized tomography [CT] scan, and normal bone scan). Any area causing suspicion of metastatic disease on bone scan was evaluated by appropriate plain radiographs and/or magnetic resonance imaging (MRI) studies of the area. Patients must have had adequate baseline organ function and coagulation factors. Patients were excluded if they ever received hormonal therapy (androgen deprivation) or had depressed hematopoietic function (platelet count, $<100{,}000/cm^3$; hemoglobin, <8.5 mg/dl; absolute neutrophil count, $<1000/cm^3$).

Vector

The vector used was an adenovirus of serotype AD5 that contains the herpes simplex virus-thymidine kinase gene and a Rous sarcoma virus long terminal repeat promoter in the region of the excised E1/E2 wild-type adenoviral genes. The adenoviral vector was constructed as described previously by Chen *et al.* (1994). It was produced in the Baylor College of Medicine gene vector laboratory, in accordance with good manufacturing practice (21 CFR210 and 211). The vector was characterized for purity and potency for clinical use. It was found to be free of adventitious contaminants, including replication-competent adenoviruses at up to 1×10^{10} viral particles (VPs). The infectious unit (IU) titer was calculated at 1×10^{11} IU/ml with a vector particle content of 2×10^{12} VPs/ml; thus, it had

an approximate VP:IU ratio of 20:1 (Nyberg-Hoffman *et al.*, 1997). Once produced, it was stored at −80°C and was diluted to the dose specified for each patient cohort.

The prodrug ganciclovir (Cytovene-Syntex, Palo Alto, CA) is Food and Drug Administration (FDA) approved for treatment of cytomegalovirus (CMV) retinitis in the immunocompromised patient and for prevention of CMV disease in transplant patients at risk. The planned dose used in this study was 5 mg/kg every 12 hr for a total of 28 doses.

Course of treatment

The predominant focus of cancer within the prostate of each patient was identified from the digital rectal examination, transrectal sonogram, and systematic needle biopsy results. Patients were admitted to the hospital on the day of vector injection. After preparation with oral antibiotics (ciprofloxacin 500 mg orally every 12 hr, 24 hr in advance), and a phosphate enema the morning of the injection, all patients were given a single 1-ml injection of vector into the prostate gland in the region judged to have the greatest concentration of tumor-bearing tissue. The dose of ADV/HSV-*tk* viral gene vector was increased for each group of patients. Group 1 (patients 8-1 through 8-4) received an injection of 1×10^8 IU of vector; group 2 (patients 9-1 through 9-5) received 1×10^9 IU of vector; group 3 (patients 10-1 through 10-4) received 1×10^{10} IU of vector; group 4 (patients 11-1 through 11-5) received 1×10^{11} IU of vector. See Table 1 for patient disease status at diagnosis and at study entry.

Parenteral ganciclovir infusions were started 24 hr after vector injection. The protocol included daily urinalysis and cultures for adenovirus in the serum, sputum, and urine, as well as serial hematologic and hepatologic studies. Vector shedding in urine was determined by DNA isolation followed by polymerase chain reaction (PCR) amplification with vector-specific primers as previously described (Timme *et al.*, 1998).

Tumor progression was evaluated by serum PSA immediately prior to the injection on day 0, and on days 2, 7, and 14; at 6 and 12 weeks; at 6, 9, and 12 months; and every 6 months thereafter. Digital rectal examinations and transrectal sonograms of the prostate were performed at 2, 6, and 12 weeks; at 6, 9, and 12 months; and every 6 months thereafer. Transrectal ultrasound-guided systematic needle biopsies of the prostate were performed at the end of the 2-week course of ganciclovir, at 3 months, and at 1 year. Pathology slides before and after treatment were interpreted by the study pathologist (T.M.W.).

Toxicity was graded according to the Cancer Therapy Evaluation Program (CTEP) common toxicity criteria published by the National Cancer Institute. All patients who experienced grade 3 or greater toxicity were immediately reported to the Institutional Review Board and to the FDA.

RESULTS

Eighteen patients were enrolled in the protocol between August 1996 and August 1997 (see patient profiles in Table 1). No patient has been lost to follow-up. Seven additional patients were evaluated but were ineligible or declined participation: three patients had metastases, one patient chose cryotherapy, two patients did not have evidence of local cancer recurrence by systematic biopsy (each had two biopsy sessions), and one patient had radiation proctitis and was unable to tolerate the transrectal ultrasound probe. The first 11 treated patients were hospitalized for the entire course of treatment. The next five patients were discharged with home health care-assisted ganciclovir infusions after they met the following criteria: (1) no ev-

TABLE 1. PATIENT DISEASE STATUS AT DIAGNOSIS AND AT STUDY ENTRY

		Diagnosis				*Study entry*				
	Patient	*Date (mo/yr)*	*Clinical stage*[a]	*Gleason grade*	*PSA*	*Age (years)*	*Date*	*Clinical stage*[a]	*Gleason grade*	*PSA*
Group 1:	8-1	12/90	T2	N/A	N/A	72	8/28/96	T2	7	4.2
Dose	8-2	1/89	T2	5	11.7	76	9/25/96	T2	7	17.8
1×10^8	8-3	1/95	T2	7	11.8	57	10/15/96	T2	7	5.7
	8-4	3/93	T2	6	6.6	71	10/15/96	T2	6	4.2
Group 2:	9-1	7/91	T2	N/A	10.7	75	11/12/96	T2	7	5.3
Dose	9-2	8/92	T2	N/A	32.4	61	11/12/96	T1c	7	7.9
1×10^9	9-3	7/93	T2	N/A	8.1	71	11/12/96	T2	7	10.5
	9-4	7/93	T2	8	23	70	12/5/96	T2	8	9.4
	9-5	10/91	T2	N/A	N/A	71	12/5/96	T2	6	3.3
Group 3:	10-1	1/90	T2	6	24.1	76	1/14/97	T2	7	6.1
Dose	10-2	10/89	T2	4	17.6	62	1/28/97	T2	7	9.2
1×10^{10}	10-3	8/92	T2	7	11.1	63	3/31/97	T2	8	3.3
	10-4	2/90	T2	5	30.3	73	4/30/97	T1c	8	10.0
Group 4:	11-1	2/91	T3	7	15.2	73	5/22/97	T2	7	12.5
Dose	11-2	5/94	T2	8	94	57	6/2/97	T2	7	13.8
1×10^{11}	11-3	6/95	T2	6	11.2	72	6/11/97	T3	7	6.5
	11-4	1/94	T3	8	19.3	69	6/26/97	T2	8	4.4
	11-5	7/87	T3	6	N/A	69	8/14/97	T2	7	13.9

[a]Stage is UICC clinical TNM stage.

TABLE 2. SUMMARY OF TOXICITY DURING PHASE I GENE THERAPY CLINICAL TRIAL FOR RECURRENT PROSTATE CANCER

Patient	*Toxicity*	*Grade*	*Outcome*
9-1	GGT (<1.5 times normal)	1	Resolved following completion of ganciclovir
10-1 and 10-2	Shaking chills	1	Resolved within 24 hr
10-4	Cellulitis	2	Resolved with intravenous antibiotics
11-1 and 11-2	Mild ALT/AST elevation	1	Resolved spontaneously following completion of ganciclovir treatments
11-3	Fever	2	Resolved with intravenous antibiotics
11-5	Fever, GGT, and LDH elevation	2	Resolved with intravenous antibiotics; ganciclovir started within 48 hr
	Total bilirubin, ALT/AST, alkaline phosphatase elevations	3	Resolved with early termination of ganciclovir (7 days)
	Thrombocytopenia	4	Resolved; required platelet transfusions for 5 days

Abbreviations: GGT, γ-Glutamyltransaminase; LDH, lactate dehydrogenase; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

idence of adenoviral vector as determined by PCR in two consecutive urine samples and (2) no evidence of toxicity.

Toxicity

No patient in group 1 experienced any toxicity; toxicity levels for the first 17 patients were grade 2 or lower (see Table 2). One patient experienced a temperature of 103.3°F (grade 2 toxicity) the night of vector injection. The patient was placed on parenteral gentamicin, ampicillin, and Flagyl (metronidazole); he promptly defervesced and completed a 1-week course of oral ciprofloxacin. All blood, urine, and nasal cultures were negative for bacterial, fungal, and viral organisms. Parenteral ganciclovir was started 72 hr after vector injection, and the patient completed the full 28-dose course without further sequelae. Another patient developed cellulitis (grade 2 toxicity) at an intravenous site that required readmission and treatment with parenteral antibiotics.

One patient (11-5) experienced grade 4 toxicity. The patient was injected with 1×10^{11} IU of adenoviral vector and developed a fever of 101.5°F early the following morning. He was placed on intravenous ampicillin, gentamicin, and Flagyl for 48 hr and promptly defervesced. A 1-week course of oral ciprofloxacin was then prescribed. All nasal, urine, and blood cultures were negative for bacterial, viral, and fungal infection, and all hematologic parameters were normal at this time. Parenteral ganciclovir infusions were started 72 hr after vector injection. On the second day of ganciclovir, the patient complained of myalgias and developed a low-grade fever of 100.2°F. Physical examination and laboratory tests remained within normal limits. On the seventh day of ganciclovir infusion, however, the patient was again febrile to 100.8°F and complained of myalgias. Tests showed thrombocytopenia of 52,000 platelets per microliter, leukocytosis of 22,000 leucokcytes per microliter, and abnormal liver function tests (alanine aminotransferase [ALT], 94 mg/dl; aspartate aminotransferase [AST],

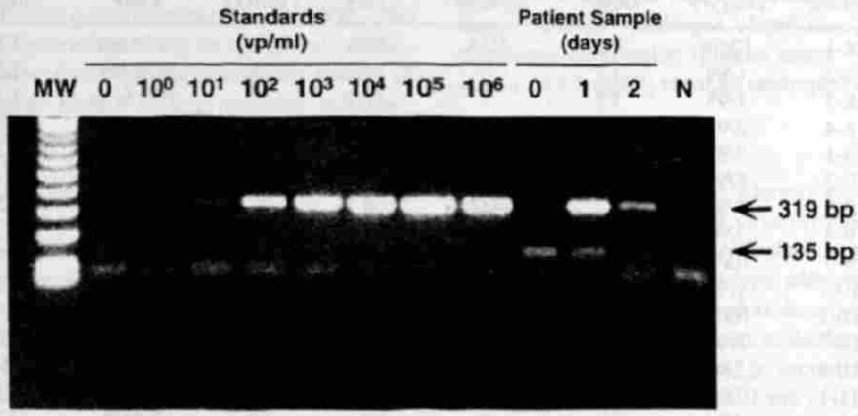

FIG. 1. Ethidium bromide-stained gel for visualization of polymerase chain reaction (PCR) products to determine extent of viral shedding in patient urine (patient 10-3). Patient sample days correspond to the day before vector delivery (day 0) and the first and second days after vector delivery. 319 bp, the size of the RSV/HSV-*tk* amplified junction for detection of vector targets; 135 bp, size of K-*ras* amplified product (internal control); N, negative control (water); vp/ml, vector particles per milliliter; MW, molecular weight standard.

117 mg/dl; alkaline phosphatase, 129 mg/dl; and lactate dehydrogenase [LDH], 1059 units per liter). The ganciclovir was discontinued, as were all other medications (gemfibrozil). The platelet count subsequently fell to 10,000 per microliter. Platelet levels were supported with five platelet transfusions for what appeared to be peripheral consumption. By day 16, the leukocyte count had returned to normal, the patient's somatic symptoms had resolved, and liver function tests and platelet count had improved.

The patient never appeared toxic and had no evidence of bleeding associated with his low platelet count. A full evaluation for sepsis and disseminated intravascular coagulopathy were negative. A CT scan of the abdomen and pelvis revealed no evidence of abdominal or pelvic pathology, and a bone marrow biopsy was normal. These findings are not typical of ganciclovir toxicity.

Vector shedding

Daily viral cultures for wild-type adenovirus in serum, ear, and nasal swabs were consistently negative in all patients.

Four or more consecutive daily urine samples for each patient were examined for the presence of adenoviral vector by PCR for the thymidine kinase gene (see Fig. 1). Vector detection in urine was dose dependent. Urine tests (test sensitivity of 10 to 100 viral particles per milliliter) were negative for the viral vector in the lowest dose group (1×10^8 IU). At 1×10^9 IU, two of five patients were positive on day 1 only (Table 3). At 1×10^{10} IU, three of four were positive, one until day 11. And at 1×10^{11} IU all five patients were positive, one until day 32.

One patient of the 18, from group 4, was able to produce a semen sample for analysis. The sample was positive for vector by PCR at 14 days postinjection.

Efficacy

Serum PSA levels (normal 0–4 ng/ml) were obtained on the day of study entry and on days 2, 5, 7, and 14, at 6 and 12 weeks; and at 6, 9, and 12 months following entry into the study. Figure 2 plots the serum PSA levels in each patient from 100 days before study entry until 1 year after, or until subsequent anticancer treatment was initiated. All patients had a rising serum PSA level before study entry. There was no immediate rise in serum PSA in response to vector injection into the prostate. No changes in PSA levels were noted at the lowest vector dose level (group 1, 1×10^8) (Fig. 2A). At 1×10^9, 1 patient (9-3, Fig. 2B) exhibited a fall in PSA levels from 10.5 ng/ml at study entry to 4.6 ng/ml 12 weeks later. Subsequently, the levels steadily rose. One patient at 1×10^{10} (patient 10-1) showed a precipitous fall from 6.1 ng/ml to a nadir of 3.0 ng/ml at 6 weeks. At the highest dose level (group 4) the first patient (11-1) showed an immediate decline from 12.5 ng/ml to a nadir of 4.8 at 12 weeks. The PSA remained less than 50% of the level at entry 1 year later (Fig. 2D). In summary, three patients (1 each from groups 2, 3, and 4) had a greater than 50% decrease in PSA levels lasting from 6 weeks to more than 1 year.

Digital rectal examination and transrectal ultrasound were performed on all patients at routine intervals. No consistent changes were noted in the size of the prostate over time. Of note was the appearance of a cystic cavity at the site of vector injection detected in patient 11-1 at 6 months postinjection (Fig. 3A and B). Systematic needle biopsies of the prostate were performed at 2 weeks, 12 weeks, and 1 year. No patient had complete histologic regression of cancer. In one patient at the highest dose level (patient 11-1) the biopsy at 2 weeks showed extensive necrosis (Fig. 3D). However, the subsequent biopsy at 12 weeks showed persistent cancer.

TABLE 3. LAST DAY OF VECTOR-POSITIVE URINE AS DETERMINED BY PCR

Patient	*Post-injection day*
8-1	Never positive
8-2	Never positive
8-3	Never positive
8-4	Never positive
9-1	Never positive
9-2	Never positive
9-3	1
9-4	Never positive
9-5	1
10-1	11
10-2	Never positive
10-3	4
10-4	6
11-1	14
11-2	9
11-3	11
11-4	32
11-5	4

Several patients have been treated for progressive prostate cancer subsequent to this phase I trial. Patients 8-3 and 8-4 had a salvage radical prostatectomy for pathologic T3N0M0 prostate cancer. The surgical margins were negative. Hormonal manipulation (androgen ablation) was initiated in three patients for rising serum PSA level (9-4), lymph node metastases on CT scan (10-2), and visceral metastases on ProstaScint (Cytogen Corporation, Princeton, NJ) scan (11-2).

DISCUSSION

This clinical trial was the result of a systematic plan to develop gene therapy for prostate cancer by our research group. Woo and colleagues modified HSV-*tk* plus ganciclovir for use with an adenoviral vector with activity when injected *in situ* into a variety of tumors in animal models (Chen *et al.*, 1994). Thompson and colleagues demonstrated activity of this form of HSV-*tk*/GCV gene therapy against mouse and human prostate cancer cell lines *in vitro* (Eastham *et al.*, 1996). Using cell lines from the mouse prostate reconstitution (MPR) model of prostate cancer, this group next demonstrated growth suppression of the local tumor and prolongation of survival in an orthotopic model system (Hall *et al.*, 1997). These effects were enhanced by concomitant androgen ablation (Hall *et al.*, 1999). Remarkably, a single course of gene therapy administered *in situ* into the tumor in the prostate also led to systemic activity against spontaneous and induced metastases (Hall *et al.*, 1997). There was no systemic toxicity or evidence of toxic effects in other genitourinary organs and no evidence of vector in sperm in these mice (Timme *et al.*, 1998).

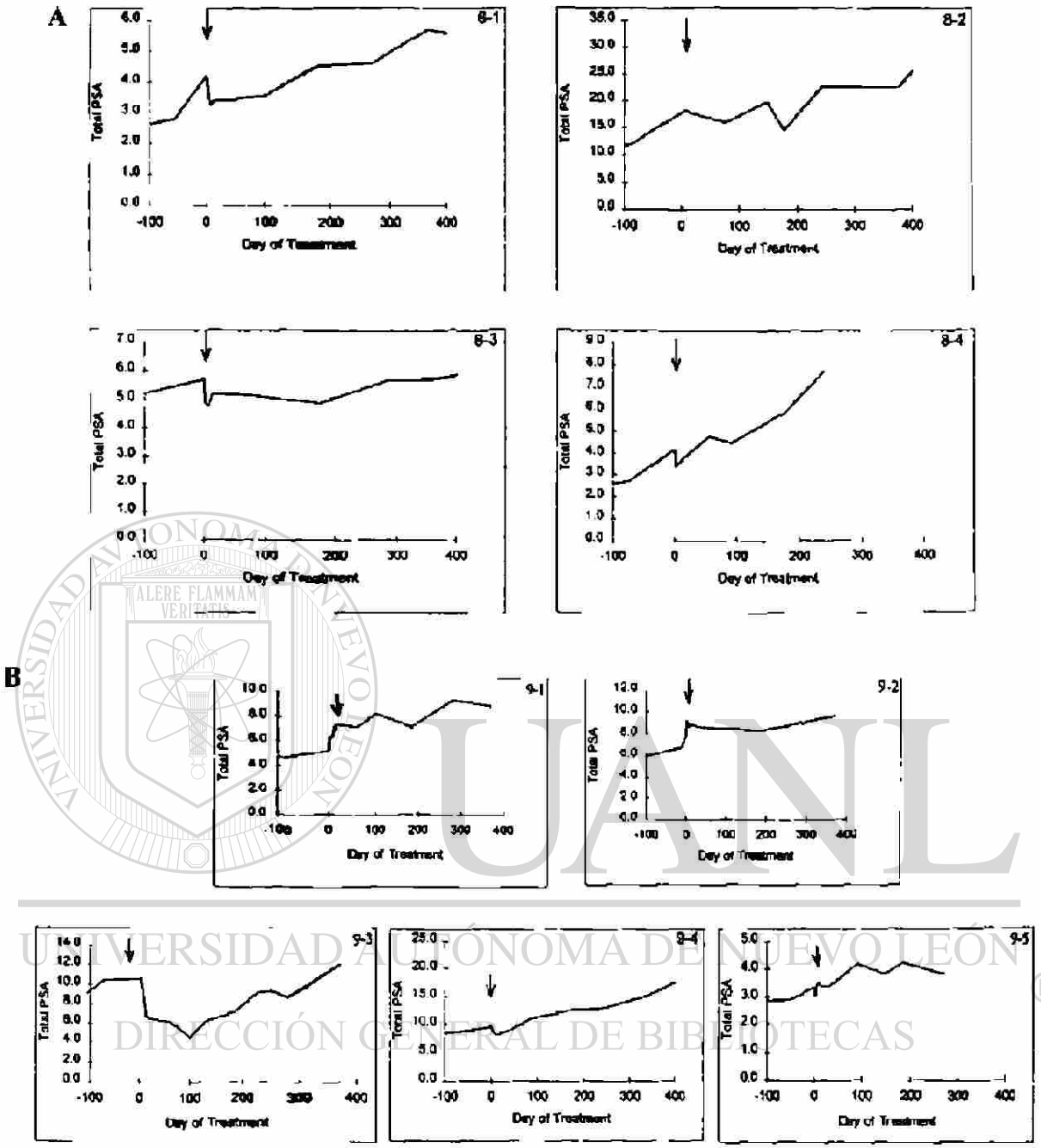

FIG. 2. Prostate-specific antigen (PSA) values of the study patients, beginning 100 days prior to study entry and continuing through the last evaluation. All values are in nanograms per milliliter. Downward arrows indicate date of injection of vector. Patient data are reported by vector dose group. All patients received a single injection of vector. A. Patients 8-1 through 8-4 received 1×10^8 infectious units (IU) vector. B. Patients 9-1 through 9-5 received 1×10^9 IU vector.

As a result of this favorable preclinical experience, we initiated a phase I dose escalation trial of ADV/HSV-*tk* with the intravenous administration of ganciclovir, selecting men with local recurrence of prostate cancer after definitive irradiation therapy for whom no widely accepted standard form of therapy is available. Alternatives include salvage radical prostatectomy or cryotherapy, which carry substantial risks, and watchful waiting or androgen ablation, which are palliative (Rogers *et al.*, 1995). The biological activity of prostate cancer in this setting can be demonstrated by a consistently rising serum PSA level and biopsy evidence of viable cancer. Local rather than distant recurrence can be verified by bone scintigraphy, abdominal and pelvic CT or MRI imaging, and in selected cases by a monoclonal antibody scan (ProstaScint). Since ADV/HSV-*tk*/GCV therapy cannot be administered systemically, we wanted to investigate the safety and efficacy of this form of gene therapy given intralesionally in men without clinically apparent metastases. With no previous information about intraprostatic injec-

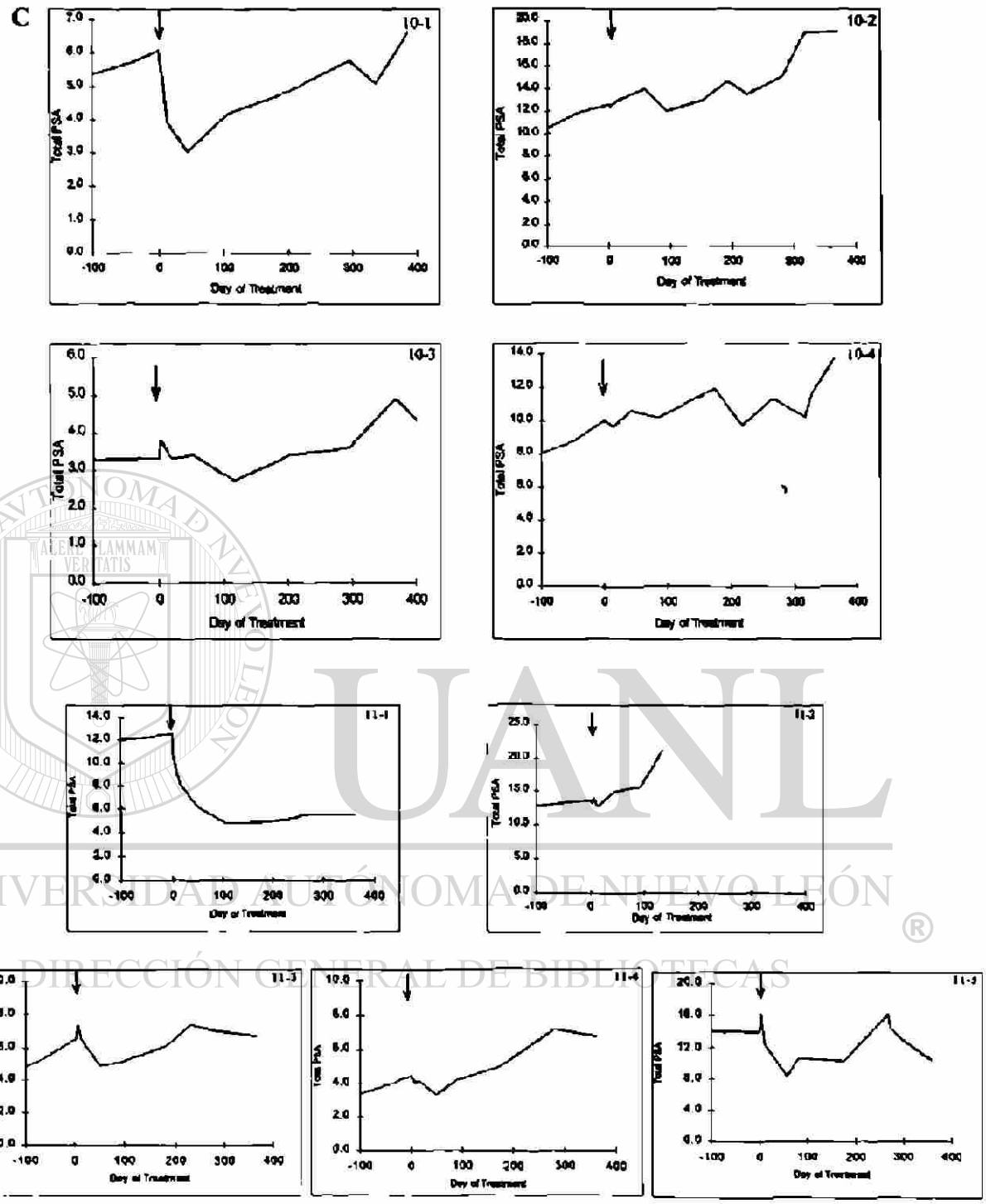

FIG. 2. *Continued.* C, Patients 10-1 through 10-4 received 1×10^{10} IU vector. D, Patients 11-1 through 11-5 received 1×10^{11} IU vector.

tions of ADV/HSV-*tk*, we did not want to treat men with newly diagnosed prostate cancers, for whom several well-defined management strategies are available (Scardino and Grayhack, 1994).

We completed the trial in 12 months, enrolling 18 patients. The regimen proved safe, with minimal toxicity in the first 17 of 18 planned patients (Table 2). One patient at the highest dose level (1×10^{11} IU) experienced grade 4 toxicity with thrombocytopenia and hepatotoxicity, possibly related to extravasation of the vector intravascularly during the injection. These complications resolved and this patient had no further sequelae. One of these patients (11-1) developed a cystic cavity within the prostate, evident sonographically in the area of the intralesional injection, that persisted during 9 months of follow-

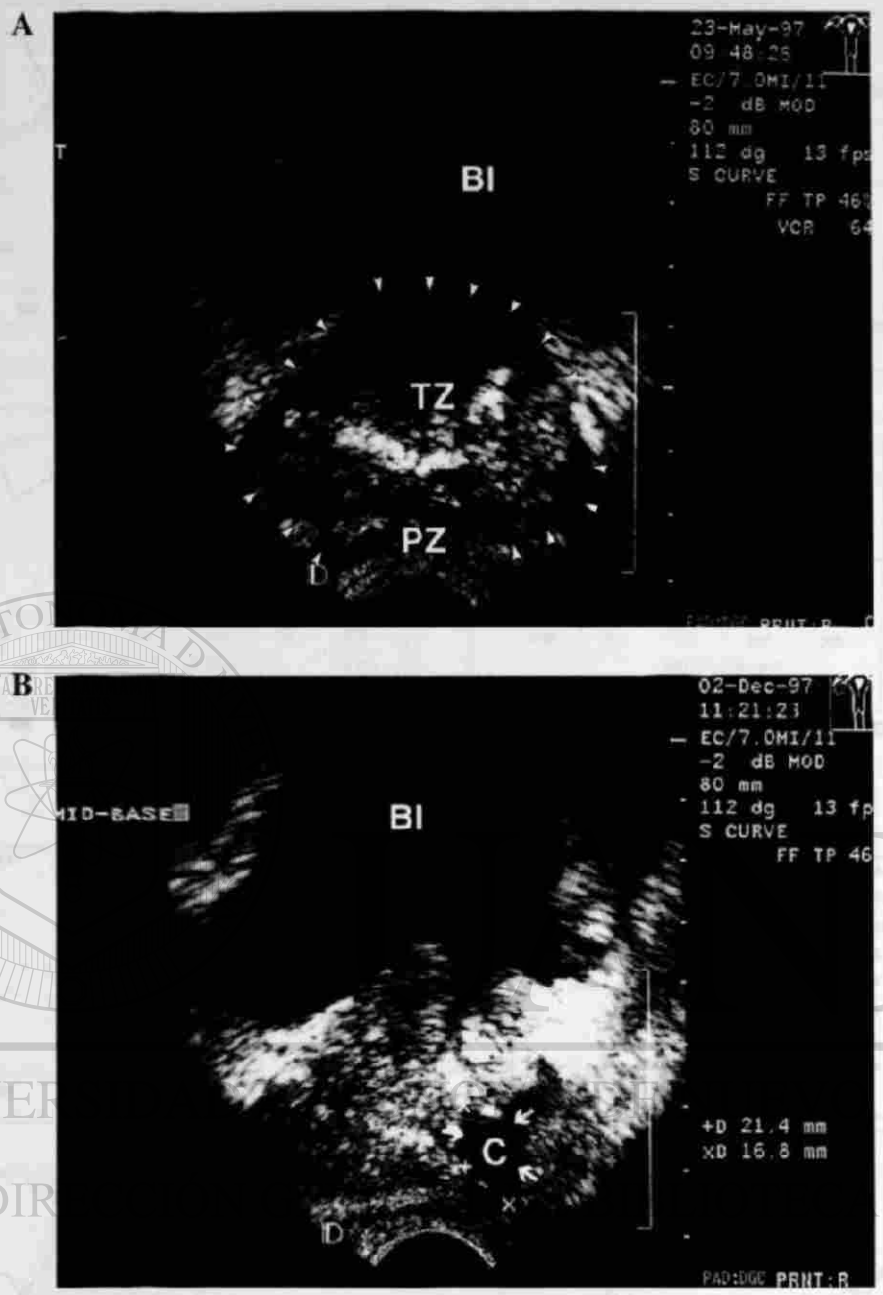

FIG. 3. Transrectal ultrasound and biopsy of patient 11-1, who had an objective response to the therapy. (Bl, Bladder; TZ, transition zone of the prostate; PZ, peripheral zone of the prostate.) **(A)** Transverse view of prostate following vector injection (arrowheads outline border of prostate). **(B)** Transverse view of prostate 6 months after therapy shows cystic cavity (C) at the site of vector injection (arrows, + and × symbols indicate cystic area).

up. Since only one of five patients experienced grade 3 or 4 toxicity at 1×10^{11} IU, we believe that dose is safe when HSV-*tk* is given as a single injection into the prostate.

HSV-*tk* plus ganciclovir has been shown to be safe in clinical trials in patients with brain tumors, ovarian cancer, and mesothelioma. We are not aware, however, of other published trials of gene therapy that show evidence of activity against human prostate cancer (Scardino *et al.*, 1998). Three patients (9-3, 10-1, and 11-1) had a decrease in serum PSA levels by more than 50% for periods ranging from 45 to 330 days despite steadily increasing PSA levels for more than 100 days before treatment (Fig. 2). No patient received any other anticancer therapy during the trial.

A decline in the serum marker PSA by more than 50% over three consecutive measurements two or more weeks apart has been associated with prolonged survival in several clinical trials of systemic therapy for hormone refractory prostate cancer (Kelly *et al.*, 1996), and seems a reasonable indicator of re-

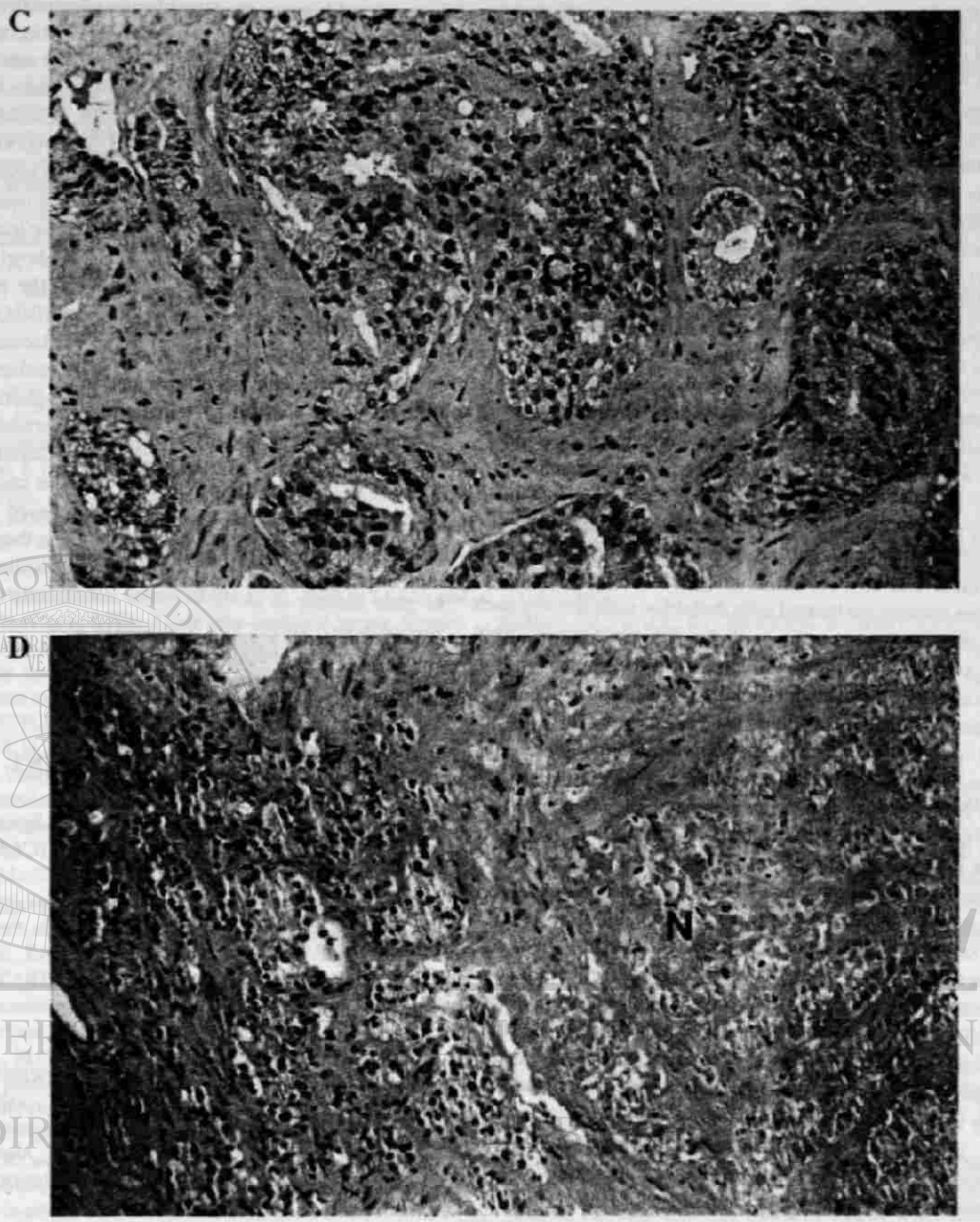

FIG. 3. *Continued.* **(C)** Photomicrograph of pretreatment biopsy of the left midportion of the prostate shows viable cancer (Ca). **(D)** Necrotic tumor tissue (N) on biopsy of the same region 2 weeks following vector injection. Original magnification: ×100.

sponse in this trial. PSA is less valid as a marker of response when antiandrogen therapy is first introduced, since this hormonal manipulation may block expression of PSA as well as reduce tumor mass (Arai *et al.*, 1990). While serial transrectal sonograms showed no consistent changes in overall prostate size over time, even among those who responded with a decrease in PSA levels, ultrasound was not sensitive enough to evaluate changes in tumor size in these patients (Egawa *et al.*, 1992). We also measured serial changes in the length of the tumor in the systematic biopsy specimens. Again, this parameter did not provide consistent results.

Despite the apparent success of this trial, there are several caveats. We did not eradicate prostate cancer from any patient. Other, well-described forms of therapy can effectively remove the local tumor in similar patients, albeit with appreciable risk of morbidity (Rogers *et al.*, 1995). We were not able to evaluate whether ADV/HSV-*tk* gene therapy had any antimetastatic effect in these patients, and it is not clear whether ADV/HSV-*tk* gene therapy will also be active against hormone-refractory prostate cancer. Also, we did not investigate the mechanism of activity in this trial. A direct, mechanical effect of intralesional injection of 1 ml seems unlikely. In our animal studies, intralesional injection of vector alone in a volume comparable to the size of the orthotopic tumor was not effective in controlled trials.

Also in this article, we do not include an evaluation of the efficacy of gene transfer or of immunological response to HSV-*tk* plus GCV therapy. We had previously reported these activities in preclinical studies using *in vivo* mouse prostate cancer models (Eastham *et al.*, 1996; Hall *et al.*, 1997). We intend to compare the efficacy of gene transfer in this selected subgroup of patients with others as well as to evaluate an extended immunological response to this gene therapy protocol and report these data at a later time. Therefore, to what extent any antitumor effect can be attributed to the direct cytotoxicity or to immunological mechanisms remains under investigation. However, it seems unlikely that the sustained response seen in patient 11-1 would have been due to direct cytotoxicity alone.

We selected for this investigational protocol patients with local recurrence of prostate cancer after definitive irradiation therapy because no standard therapy is widely considered safe and effective in this situation. About one-third of men treated for clinically localized prostate cancer each year are treated with definitive radiotherapy. In 1998, 184,500 new cases of prostate cancer are expected to be diagnosed (Landis *et al.*, 1998); about 80% of these will be localized at diagnosis. More than 50,000 men in this country are treated with definitive radiotherapy each year. Of these, at least one-third will develop progression within 5 years, and about half of these will have local recurrence only with no evidence of metastases (Kupelian *et al.*, 1997). Thus, we estimate that some 9000 patients per year face the dilemma of local recurrence of cancer after definitive radiotherapy.

Prostate cancer, among the solid tumors, may be particularly suitable for gene therapy. The organ is expendable after reproductive needs are met, the primary tumor is readily accessible both for intralesional injections and for repeat biopsies, and a well-characterized circulating marker of response (PSA) is readily available. Gene therapy appears to cause little morbidity and could be applied repeatedly to palliate these slow-growing cancers long enough for patients to live out their natural lives even if the cancer is not completely eradicated. In addition, limited, occult micrometastases—the major cause of treatment failure—may be particularly susceptible to gene therapy strategies that augment the immunogenicity of tumor cells and thereby elicit systemic anti-tumor immunity (Hall *et al.*, 1997, 1999). Intraprostatic injections can be administered without anesthesia in an outpatient sonography room, ganciclovir can be administered intravenously in an outpatient infusion center, and the patients can be monitored in the outpatient clinic; thus the expense of this therapeutic regimen is minimal. If this form of gene therapy can be shown to be effective in larger phase II trials, other possibilities for its use might include treatment in conjunction with androgen ablation before radiation therapy, and primary treatment for low-risk cancers in young men or for high-risk cancers in older men or those with serious comorbid conditions.

While we saw some evidence of acitivity in this phase I trial, intralesional injection at a single site is unlikely to provide adequate control of many prostate cancers. Since cancer of the porstate is multifocal, and the location of cancer within the prostate cannot be determined accurately, optimal therapy will likely require a uniform distribution of the vector throughout the gland. Consequently, we have extended the clinical trial to explore the safety of multiple sites of injection and repeat injections. Since the distribution of the vector around a line of injection is not known, we also plan to administer two different doses of the vector alone several days before radical prostatectomy to examine its distribution within the removed prostate. In an additional trial, we also plan to treat men with high-risk cancers (defined by their clinical stage, Gleason grade, and serum PSA level) (Kattan *et al.*, 1998), with ADV/HSV-*tk*/GCV gene therapy 4–6 weeks prior to radical prostatectomy. The distribution of necrosis and apoptosis within the cancer will be determined along with a detailed analysis of the local immune response. These studies should allow us to develop an optimal strategy for delivery of the vector within the prostate. Additional preclinical studies should also establish a rationale for the use of ADV/HSV-*tk*/GCV therapy in combination with other gene therapy approaches designed to enhance and/or sustain the local and possible systematic therapeutic effects.

In conclusion, gene therapy via an intraprostatic injection of ADV/HSV-*tk* with the intravenous administration of the prodrug GCV is safe in patients with prostate cancer. Early evidence of efficacy suggests we should proceed with trials that will evaluate the utility of this form of gene therapy as primary or adjuvant treatment for prostate cancer. While no single form of gene therapy is likely to prove sufficiently efficacious, our favorable experience with this form of gene therapy for prostate cancer may have implications for the treatment of other human malignancies.

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Thymidine kinase (TK) gene therapy of solid tumors: valacyclovir facilitates outpatient treatment.

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BACKGROUND: In a Phase I study replication-deficient adenovirus containing the herpes simplex virus (HSV) thymidine kinase (TK) gene (AdV-HSV-TK) was instilled intraperitoneally in patients with recurrent ovarian cancer. Patients were treated with Acyclovir (ACV) or Valacyclovir (VCV) as enzymatic substrates. The purpose of this study was to compare serum levels of ACV and VCV. PATIENTS AND METHODS: The antiherpetic prodrug and Topotecan (1.0 mg/m2 over 30 minutes each day for 5 days) were started 24 hours after vector application. Eight patients received ACV (15 mg/kg i.v. over one hour every 8 hours for 42 doses), two patients were started on ACV for 5 days and then switched to oral VCV (2 g every 8 hours for a total of 42 doses). Blood samples were obtained 20 minutes prior to each drug. RESULTS: Serum levels of ACV and VCV (converted to ACV) were comparable. CONCLUSIONS: Suicide gene therapy with TK is under investigation in a

variety of solid tumors. Replacing ACV by VCV will offer a cost-effective alternative and will significantly reduce duration of hospital stay improving quality of life and facilitating an outpatient gene therapy concept.

Publication Types:

- Clinical trial
- Clinical trial, phase i
- Controlled clinical trial

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Abstract

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TRIAL

Phase I Study of Adenoviral Delivery of the HSV-tk Gene and Ganciclovir Administration in Patients with Recurrent Malignant Brain Tumors

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Between December 1996 and September 1998, 13 patients with advanced recurrent malignant brain tumors (9 with glioblastoma multiforme, 1 with gliosarcoma, and 3 with anaplastic astrocytoma) were treated with a single intratumoral injection of 2×10^9, 2×10^{10}, 2×10^{11}, or 2×10^{12} vector particles (VP) of a replication-defective adenoviral vector bearing the herpes simplex virus thymidine kinase gene driven by the Rous sarcoma virus promoter (Adv.RSVtk), followed by ganciclovir (GCV) treatment. The VP to infectious unit ratio was 20:1. Our primary objective was to determine the safety of this treatment. Injection of Adv.RSVtk in doses $\leq 2 \times 10^{11}$ VP, followed by GCV, was safely tolerated. Patients treated with the highest dose, 2×10^{12} VP, exhibited central nervous system toxicity with confusion, hyponatremia, and seizures. One patient is living and stable 29.2 months after treatment. Two patients survived >25 months before succumbing to tumor progression. Ten patients died within 10 months of treatment, 9 from tumor progression and 1 with sepsis and endocarditis. Neuropathologic examination of postmortem tissue demonstrated cavitation at the injection site, intratumoral foci of coagulative necrosis, and variable infiltration of the residual tumor with macrophages and lymphocytes.

Key Words: **gene therapy; HSV-tk; ganciclovir; glioblastoma; astrocytoma; adenovirus; stereotaxic technique.**

INTRODUCTION

The American Cancer Society estimates that 16,800 individuals will develop and 13,100 individuals will die from primary tumors of the nervous system in 1999, with the majority of these cases being malignant gliomas (1). Although there have been significant advances in neurosurgery, neuroradiology, radiation therapy, and medical oncology, malignant brain tumors remain resistant to all current therapies, and survival is often less than 1 year (2–4). Even when conventional treatments extend survival, malignant brain tumors almost invariably recur because of surviving tumor cells that have invaded the surrounding brain. All treatments must balance effective tumor killing against toxicity to surrounding neural tissue. The prognosis after recurrence of a malignant brain tumor is particularly poor (3, 5).

Transduction of tumor cells with the herpes simplex virus thymidine kinase (HSV-tk) gene, which activates the nucleoside analog prodrug ganciclovir (GCV), has been one of the most effective approaches in treating experimental brain tumors (6–12). This therapy selectively kills dividing cells, an advantageous feature in the brain where most normal cells are not actively dividing. Treatment efficacy may be enhanced by the "bystander effect," whereby surrounding nontransduced tumor cells are also killed through a variety of mechanisms which are believed to include transfer of toxic metabolites via gap

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junctions (13–15), induction of apoptosis (16), and activation of a host immune response (17–19).

Toxicity studies of intracerebral injection of an adenoviral vector, followed by GCV treatment, in cotton rats (20), which are permissive to adenoviral infection, and in other rodent models (7, 10, 21) have shown no clinical or systemic pathological sequalae. Our and other centers have also conducted preclinical toxicity studies in primates (21–23). Although clinical symptoms have not been observed following intracerebral injection of the vector at lower doses, dose-dependent toxicity has been observed in animals receiving higher doses of the vector and GCV (21, 22). The goal of the present study was to determine the safety of Adv.RSVtk + GCV treatment for recurrent advanced malignant brain tumors in humans and to determine the maximal safely tolerated vector dose for intratumoral injection at a single site.

MATERIALS AND METHODS

Permission for this Phase I clinical trial was granted by the Baylor College of Medicine Institutional Review Board (IRB), the Food and Drug Administration (FDA), and the National Institutes of Health Recombinant DNA Advisory Committee (RAC) on the basis of the efficacy and toxicity studies we performed in animal models (7–10, 20, 22) and after production and testing of clinical grade adenoviral vector. Adverse events and patient deaths were reported to the IRB, FDA, and RAC according to the recommended guidelines.

Patient Selection

Adults (≥18 years of age) with recurrent and clinically progressive malignant glial or metastatic tumors were eligible for enrollment. Histological confirmation of the diagnosis was required, and tumors had to be identifiable on neuroimaging studies. Subjects eligible for the study had received and failed conventional treatments, including surgery and radiation therapy. A Karnofsky performance score ≥60 was required. In addition, adequate systemic organ function was required as determined by the following criteria: serum creatinine of ≤1.5 mg/dl or creatinine clearance ≥45 ml/min/m^2, platelet count ≥100,000/mm^3, absolute neutrophil count ≥8.5/mm^3, normal PT and aPTT, and bilirubin ≤2.5 mg/dl.

Specific exclusion criteria included acute infection (including HIV infection), progressive systemic malignancy, or unacceptable anesthesia risk. Patients with tumors with cavities likely to communicate with either a subarachnoid space or the ventricular system were excluded. Patients with tumors causing significant mass effect or increases in intracranial pressure and requiring surgical debulking were not eligible. Pregnant patients were excluded, and female subjects of child-bearing age were required to practice birth control for the duration of the study. Patients were informed that the treatment was experimental, and detailed informed consent was obtained at enrollment.

Prior to the gene therapy treatment, each patient underwent a complete history and physical examination, laboratory testing, magnetic resonance imaging (MRI) of the brain, and neuropsychological testing. The neuropsychological test battery was tailored to each patient's level of functioning, area of brain involvement, and endurance, with the goal being detection of significant changes from the baseline examination. Tumor histology was reviewed by the study neuropathologist (J.C.C.) to reconfirm tumor diagnosis. Pretreatment presence of serum-neutralizing antibodies to wild-type adenovirus and of adenovirus in serum, urine, and nasal mucosa was determined for each patient.

Creation of the Adv.RSVtk Vector

Construction of the replication-deficient viral vector has been described in detail previously (10, 24). The adenovirus E1A region was replaced with the HSV-tk gene under the control of the Rous sarcoma virus (RSV) long-terminal repeat and grown in 293 cells. The vector was produced at the Baylor College of Medicine Gene Vector Laboratory using Good Laboratory Practices. In addition to standard quality control procedures used in vector production, we verified that the vector preparation did not contain measurable wild-type contamination or E1A recombination from 293 cells by polymerase chain reaction (PCR) for the wild-type E1A sequence. The vector preparation was certified to be free of replication-competent adenovirus at <1 per 10^{10} VP. Additionally, the failure of cotton rats to sustain productive adenoviral infections after intracerebral delivery of the vector and the failure of adenoviral proliferation on HeLa cells in culture established that there was no significant replication-competent viral contamination. The sensitivity of the HeLa culture assay was one wild-type genome per 75,000 vector genome equivalents.

Adv.RSVtk Vector Dose

Based on our previous toxicity trials (22), we designed a dose-escalation protocol to test the safety of four Adv.RSVtk vector doses: 2×10^9, 2×10^{10}, 2×10^{11}, and 2×10^{12} VP. The VP to infectious unit (IU) ratio, measured using the cytopathic effect (CPE) assay (25), was determined to be 20:1. A minimum of two patients were injected with each dose, beginning with the lowest dose, 2×10^9 VP (1×10^8 IU); if no toxicity was observed over the next month, subsequent patients received the next higher dose.

Injection of the Adv.RSVtk Vector

A Leksell stereotactic frame was applied to the patient's skull using local anesthesia and monitored anesthesia care. Tumor coordinates were generated after computerized tomography (CT) or MRI with the Leksell frame in place. Patients were returned to the operating room, and an 18-gauge stainless steel injection cannula was directed to the target through a twist-drill craniostomy or burr hole. One milliliter of vector solution was injected through the cannula over 5 min. The cannula was left in place for an additional 5 min and then withdrawn. A central venous catheter was placed in most patients to facilitate subsequent intravenous access.

Patients were monitored in the neurosurgical intensive care unit and then transferred to a private room on the neurosurgical unit. A postoperative MRI was obtained within the first 24 h to confirm injection placement and to observe if swelling or hemorrhage had occurred. Standard perioperative medications were administered, including antibiotics, dexamethasone, anticonvulsants, and analgesics.

Ganciclovir Treatment

Twenty-four hours after vector injection, intravenous GCV administration was begun, using a dose of 5 mg/kg over 1 hour, every 12 h, for a total of 28 doses over 14 days. During GCV treatment, patients underwent daily physical and neurological examinations and frequent laboratory studies including complete blood counts, electrolyte levels, and liver function tests. Serum, urine, and nasal swab samples were monitored for vector shedding, and serum was tested for neutralizing antibodies.

Toxicity following vector injection and during GCV treatment was graded using the Common Toxicity Criteria published by the Cancer Therapy Evaluation Program of the National Cancer Institute (26). Permanent Grade 3 or recurrent Grade 4 toxicity was the criterion for cessation of treatment.

The GCV (Cytovene) used in this trial was manufactured by Syntex Corp. (Palo Alto, CA). The drug is approved for the treatment of cytomegalovirus (CMV) retinitis in immunocompromised patients and for the prevention of CMV disease in transplant patients at risk for CMV disease.

Posttreatment Assessment

Patients underwent a neuropsychological evaluation approximately 2 weeks after vector injection. After discharge from the hospital, patients were seen as outpatients every 2 weeks for the first 2 months and then monthly for the first year. Physical examination and laboratory studies were obtained at each visit. A second neuropsychological evaluation was performed at 2 months postinjection. MRI scans were obtained in the first week after completion of HSV-tk/GCV treatment and every 12 weeks

TABLE 1

Patient Pretreatment Characteristics

PT	Age at DX (years)	Age at GT (years)	DX-GT period (months)	Sex	Tumor path	Side	Loc	KFS %	No. Pretreatment resect	XRT	Chem
1	33.2	34.8	19.3	M	GBM	L	Fr	60	1	Y	Y
2	46.2	52.4	74.6	M	AA	R	P	60	3 + 1P	Y	Y
3	57.2	57.8	7.3	M	GBM	L	O	70	2	Y*	Y
4	42.4	53.8	136.7	M	AA	R	FrT	80	1	Y*	Y
5	67.3	68.6	15.7	M	GBM	R	Fr	60	1	Y	YY
6†	41.0	41.8	9.8	F	GBM	L	Fr	60	2	Y*	YY
7	38.3	39.3	12.2	M	GBM	L	P	90	2 + 1P	Y + 1P	Y
8	64.1	65.3	14.9	M	GS	B	Fr	80	2	Y	N
9	64.3	64.9	7.5	M	GBM	R	T	60	1	Y	Y
10	47.6	48.5	10.7	M	GBM	R	PO	80	3	Y	N
11	46.2	50.0	44.6	M	GBM	L	T	80	2	Y	YY
12	37.3	38.3	12.3	F	GBM	R	P	80	1	Y	Y
13	43.9	45.0	13.6	M	AA	L	Fr	60	2	Y	Y
	Mean	Mean	Mean	11 M	9 GBM	7 L	5 Fr	6 60%	2 3r	13 Y	11 Y
	48.4 ± 11.2	50.8 ± 11.0	29.2 ± 34.4	2 F	1 GS	6 R	3 P	1 70%	6 2r		2 N
					3 AA		2 T	5 80%	5 1r		
							1 O	1 90%			
							1 FrT				
							1 PO				

Note. Abbreviations used: AA, anaplastic astrocytoma; B, bilateral; Chem, chemotherapy; DX, diagnosis; F, female; Fr, frontal; FrT, frontotemporal; GBM, glioblastoma multiforme; GS, gliosarcoma; GT, gene therapy; KFS, Karnofsky scale; L, left; Loc, tumor location; M, male; N, no; O, occipital; P, parietal; PO, parietooccipital; PT, patient; DX-GT period, time between tumor diagnosis and GT; r, resect, resections; R, right; T, temporal; XRT, X-radiation treatment; Y, yes; YY, two courses of chemotherapy; ±, standard deviation; *, subsequent XRT boost to tumor; +1P, also underwent a post-GT resection or XRT; †, patient diagnosed and treated (resection, radiation, and chemotherapy) for oligodendroglioma 4 years before GBM, for which she also underwent resection, radiation (focused), and chemotherapy.

thereafter. The MRIs were read independently by a neuroradiologist and by a neurosurgeon. Tumor response was monitored by clinical criteria and by MRI. Palliative and therapeutic options were discussed and made available to patients who deteriorated due to tumor progression.

Neutralizing serum antibodies to adenovirus type 5 (AV5) were determined before and at 14, 28, 42, 56, and 84 days after vector injection in most patients and thereafter at outpatient follow-up visits in patients with extended survival. The assay used to measure AV5-specific neutralizing serum antibodies has been described in detail previously (20). Neutralizing antibody titers were expressed as the $\log_2$ of the reciprocal of the last dilution of antiserum that inhibited virus-induced cytopathic effects by 100%.

Serum, urine, and nasal swabs were obtained at 1, 2, and 4 weeks after vector injection and examined by plaque assay for evidence of adenovirus shedding. A detailed description of the plaque assay has been published previously (20). Minimum detection in these assays was 20 plaque-forming units (pfu).

The presence of viral vector DNA in urine was determined by PCR and Southern blot hybridization of the PCR product. The sensitivity was 10 VP/ml of urine. Urine was analyzed daily for vector DNA during hospitalization and at 2 and 6 weeks after vector injection. The procedure used has been previously described (22).

RESULTS

Thirteen patients were enrolled into the study between December 1996 and September 1998 (Table 1). The mean age was 50.8 years (range 34.8 to 68.6 years). There were 11 males and 2 females. Pretreatment Karnofsky scores ranged from 60 to 90. All patients had primary central nervous system neoplasms. Tumor histologies were nine glioblastoma multiformes (GBM), one gliosarcoma, and three anaplastic astrocytomas (AA). The mean intervals between initial diagnosis and adenoviral treatment for patients with a GBM or GS were 15.5 (±SD 11.6) and 14.9 months, respectively; for patients with an AA the mean interval between initial diagnosis and Adv.RSVtk treatment was 74.9 (±SD 61.5) months.

All patients had undergone previous tumor resection and had received conventional external beam radiation therapy. Eleven of the 13 patients had received chemotherapy, and 2 patients had received additional chemotherapy after tumor recurrence. Three patients had received conformal radiation boosts, with 2 of the 3 receiving the boost after tumor recurrence. Eight patients had undergone reoperation for tumor debulking.

Tumor characteristics on imaging were typical of advanced recurrent gliomas, with complex configurations, cystic areas, and brain invasion. Quantitative descriptions are difficult in this setting, but the cross-sectional diameter was greater than 3 cm in the majority of patients.

Clinical Course during Treatment

Table 2 summarizes the clinical courses of all patients enrolled. The sequence of dose escalation was as follows: 2 patients each received 2×10^9 and 2×10^{10} VP; 3 patients received 2×10^{11} VP; and 2 patients received $2 \times$

TABLE 2

Gene Therapy and Outcome

PT	VP	Cog (2 weeks)	GT Tox	Postoperative adverse events (days 1–14)	Post-DX survival (months)	Post-GT survival (months)
1	2×10^9	—	N	Perioperative focal seizures, small intratumoral hemorrhage	47.6	28.4
2	2×10^9	↓	N	Slight rash	78.2	3.5
3	2×10^{10}	—	N		11.3	4.0
4	2×10^{10}	—	N		162.6	25.9
5†	2×10^{11}	↓	N	Increased left hemiparesis, thrombocytopenia	16.8	1.1
6	2×10^{11}	—	N	Seizure day 6	40.0**	29.2**
7	2×10^{11}	↑	N	Thrombocytopenia	21.5	9.3
8	2×10^{12}	↓↓*	Y	Lethargy, confusion, mild hyponatremia, fever, leukocytosis, intratumoral hemorrhage day 6	16.9	2.0
9^	2×10^{12}	↓↓*	Y	Air in ventricle, lethargy, confusion, fever, hyponatremia, leukocytosis, increased CSF protein, hydrocephalus	10.1	2.6
10	2×10^{11}	↓	N		16.2	5.5
11	2×10^{11}	↓	N	Increased right hemiparesis, lethargy	45.7	1.1
12	2×10^{11}	—	N	Thrombocytopenia, increased liver enzymes	14.3	2.0
13	2×10^{11}	—	N	Increased right hemiparesis, hyponatremia	20.9	7.4
					M 38.4 ± 42.1 (median 20.9)	M 9.2 ± 10.5 (median 4.0)

Note. Abbreviations used: Cog, change in cognition from baseline at 2 weeks after Adv.RSVtk injection; DX, diagnosis; GT, gene therapy; M, mean; N, no; Tox, toxicity; VP, vector particles (20:1 VP:IU ratio); Y, yes; ↑, slightly better; —, same; ↓, slightly worse; ↓↓, worse; ^, completed 4 of 28 GCV treatments; †, received 25 of 28 GCV treatments; *, unable to undergo formal testing due to neurological decline; **, patient is alive at time of publication.

10^{12} VP. After evidence of toxicity at 2×10^{12} VP, the 4 subsequent patients were treated with 2×10^{11} VP. Eleven of the patients completed the full 2-week course of treatment with GCV. Patient 9 received 4 of the 28 GCV doses. Patient 5 received 25 of the 28 GCV doses.

No patient who received 2×10^9 VP ($N = 2$) or 2×10^{10} VP ($N = 2$) experienced any significant treatment-related toxicity other than brief perioperative focal seizures in patient 1 who had a known seizure disorder at the time of enrollment. This patient also developed a small intratumoral hemorrhage that resolved without treatment. Patient 2 developed a slight rash that may have been caused by the GCV. The rash resolved without treatment and without interruption of the GCV treatment.

In the 2×10^{11} VP group ($N = 7$), three patients (patients 5, 11, and 13) experienced a mild increase in preexisting hemiparesis during GCV treatment. Patient 13 also developed transient hyponatremia (131 meq/liter on day 7 after vector injection). This patient had required Na tablets for hyponatremia prior to gene therapy. Patient 12 developed thrombocytopenia (platelet count = 132,000/mm^3 on day 13), and the platelet counts of two patients (patients 5 and 7) with low baseline platelet counts (132,000 and 144,000/mm^3, respectively) declined during GCV treatment (101,000/mm^3 on day 11 and 78,000/mm^3 on day 12, respectively). Platelet counts returned to baseline following GCV treatment; platelet transfusion was not required.

Three (patients 5, 11, and 12) of the seven patients who received 2×10^{11} VP had rapidly deteriorating clinical courses (patient 5 withdrew from the study near the completion of GCV treatment) and died within 2 months of gene therapy. There was no evidence of treatment-related toxicity in these three patients. Patients 5 and 11 had evidence of rapid tumor progression that continued during treatment. Patient 12 developed pneumonia 5 weeks after injection, followed by disseminated intravascular coagulation (DIC), adult respiratory distress syndrome (ARDS), sepsis, and multiorgan failure. She died 2 months after vector injection; postmortem examination revealed marantic endocarditis.

Both patients treated with 2×10^{12} VP of the Adv.RSVtk vector demonstrated clinically significant toxicity during the treatment period. The first patient (patient 8) had been treated for a left frontal gliosarcoma that had recurred in the right frontal lobe after two previous resections. Twenty-four hours after injection of the vector into the right frontal tumor mass, the patient exhibited confusion and lethargy. He improved and GCV was started on day 2. Six days after vector injection, the patient had a generalized seizure, and a CT scan demonstrated a right frontal hematoma. The patient improved after the postictal period, but did not return to his neurological baseline. He had hyponatremia (Na = 129 meq/liter on day 12), leukocytosis, and low-grade fever (38.7°C on day 11). He improved, completed GCV treatment, and was discharged from the hospital. He remained stable after discharge until 6 weeks after vector injection when he presented with status epilepticus. He did not regain consciousness. A CT scan demonstrated severe bifrontal edema suggestive of tumor progression. The patient's family asked that care be withdrawn. He died 2 months after vector injection. Post-

mortem examination of the brain showed cavitation in the right frontal lobe along the injection tract, surrounding edema, and mass effect. Gross residual tumor was seen in both frontal lobes. The residual right frontal tumor was heavily infiltrated with lymphocytes, and foci of coagulative necrosis were present. Scattered perivascular lymphocytes were seen in the white matter of the injected hemisphere and in the pons, and there was also patchy denudation of the ependyma on the injected side. No demyelination or macrophage infiltration was seen in association with the perivascular lymphocytes. There was no evidence of brain herniation.

The second patient (patient 9) who was treated with 2×10^{12} VP had a recurrent right temporal GBM. He complained of a severe headache in the immediate postinjection period, and a CT scan demonstrated air within the ventricular system, raising the possibility of vector entry into the cerebrospinal fluid. Twelve hours after the injection the patient became obtunded, was unable to follow commands, and developed a fever of 38.9°C. He subsequently recovered rapidly to his neurological baseline, and GCV treatment was initiated. Three days postinjection the patient developed hyponatremia (Na = 126 meq/liter) and obtundation. Ganciclovir treatment was stopped. He initially improved after correction of the hyponatremia, but later deteriorated. A CT scan demonstrated hydrocephalus, and a ventriculoperitoneal shunt was placed on day 14. CSF protein was found to be markedly elevated (624 mg/dl). The patient subsequently gradually improved until tumor progression supervened. The patient died 2.6 months after vector injection. An autopsy was not performed.

One patient (patient 6) with a GBM is alive 29.2 months after vector injection. She is clinically stable and her left frontal tumor has changed little over the 2-year period (Fig. 1). Two other patients, one with a GBM (patient 1) and one with an AA (patient 4), survived for 28.4 and 25.9 months, respectively. A transient reduction in tumor dimensions was observed in patient 4, and both patients had a posttreatment interval of reduced steroid requirements. Survival times to date range from 1 to 29.2 months (median 4.0, mean 9.4 ± SD 10.8 months) (Fig. 2).

Neuropathologic examination of six brains from patients who received 2×10^{10} VP (patients 3 and 4), 2×10^{11} VP (patients 7, 10, and 12), or 2×10^{12} VP (patient 8) disclosed recurrent viable malignant glioma in all specimens. Minimal to moderate intratumoral inflammation and foci of coagulative necrosis were observed in all specimens. As noted earlier, minimal extratumoral inflammation was seen in the brain of patient 8, who received the highest vector dose. Patient 7, who received 2×10^{11} VP, underwent tumor debulking at 2 months and died 9 months after vector injection. Neuropathologic examination of the resected tumor at 2 months showed heavy macrophage and lymphocyte infiltration and areas of coagulative necrosis within the GBM. No inflammation was observed in more viable areas of the tumor. At autopsy, considerable coagulative necrosis was found in the recurrent tumor but minimal intratumoral inflammation. No inflammation was seen in the brain outside of the tumor bed. Detailed neuropathologic studies will be reported separately.

No adenovirus was detected by plaque assay in any of the serum, urine, or nasal swab samples obtained after vector injection or in any of the urine samples examined by PCR.

Posttreatment elevations in adenoviral antibody titers, relative to pretreatment levels, were detected in 10 of the 12 patients studied (Fig. 3A). In 3 patients with extended survival whose antibody titers were followed for more than 100 days after vector injection (Fig. 3B), 2 patients (patients 4 and 6) maintained significantly elevated titer levels. The third patient (patient 1) had titer levels around baseline throughout his treatment.

Neuropsychological testing was performed shortly before vector injection in all patients, at 2 weeks (at the completion of GCV treatment) in 11 of the 13 patients (testing could not be performed for patients 8 and 9), and at 2 months after vector injection in 6 of the 9 patients alive at 2 months. Because of significant differences in the patients' cognitive abilities and because of the deteriorating clinical course of most of the patients, meaningful neuropsychological data for the group as a whole could not be obtained. Comparison of each patient's performance before and at the end of GCV treatment (Table 2) identified no obvious treatment-associated effect on neuropsychological performance.

Discussion

Genetic approaches to treating neoplasia have included the transduction of tumor cells with genes or gene products that activate cytotoxic prodrugs (27), upregulation of tumor suppressor genes such as p53 (28, 29), stimulation of immune response (30–32), and enhancement of cellular response to other therapies such as radiation (33, 34).

A number of viral and nonviral vectors are available for gene delivery. Experimental brain tumors have been successfully treated with retroviral (35–39) and adenoviral (6, 10–12, 28) vectors, and clinical trials using these approaches to treat malignant brain tumors have been and are being conducted (40–45). Adenoviral vectors are attractive for use in gene therapy because of their efficiency in transducing many cell types, ease of high titer production, episomal genome location, and ability to transduce nondividing cells.

Our Phase I trial examined the safety of the injection of Adv.RSVtk into a single intratumoral site, followed by intravenous GCV, in patients with advanced malignant brain tumors. Pre- or posttreatment tumor resection was not included as a part of this protocol although two patients underwent postinjection tumor debulking because of tumor growth. Multiple or repeat vector injections were not carried out. The trial was designed in this manner for the following reasons: (i) the simple design offered the best opportunity to establish the safe/toxic dose levels, (ii) any treatment effect would not be confounded with other treatments, and (iii) the design com-

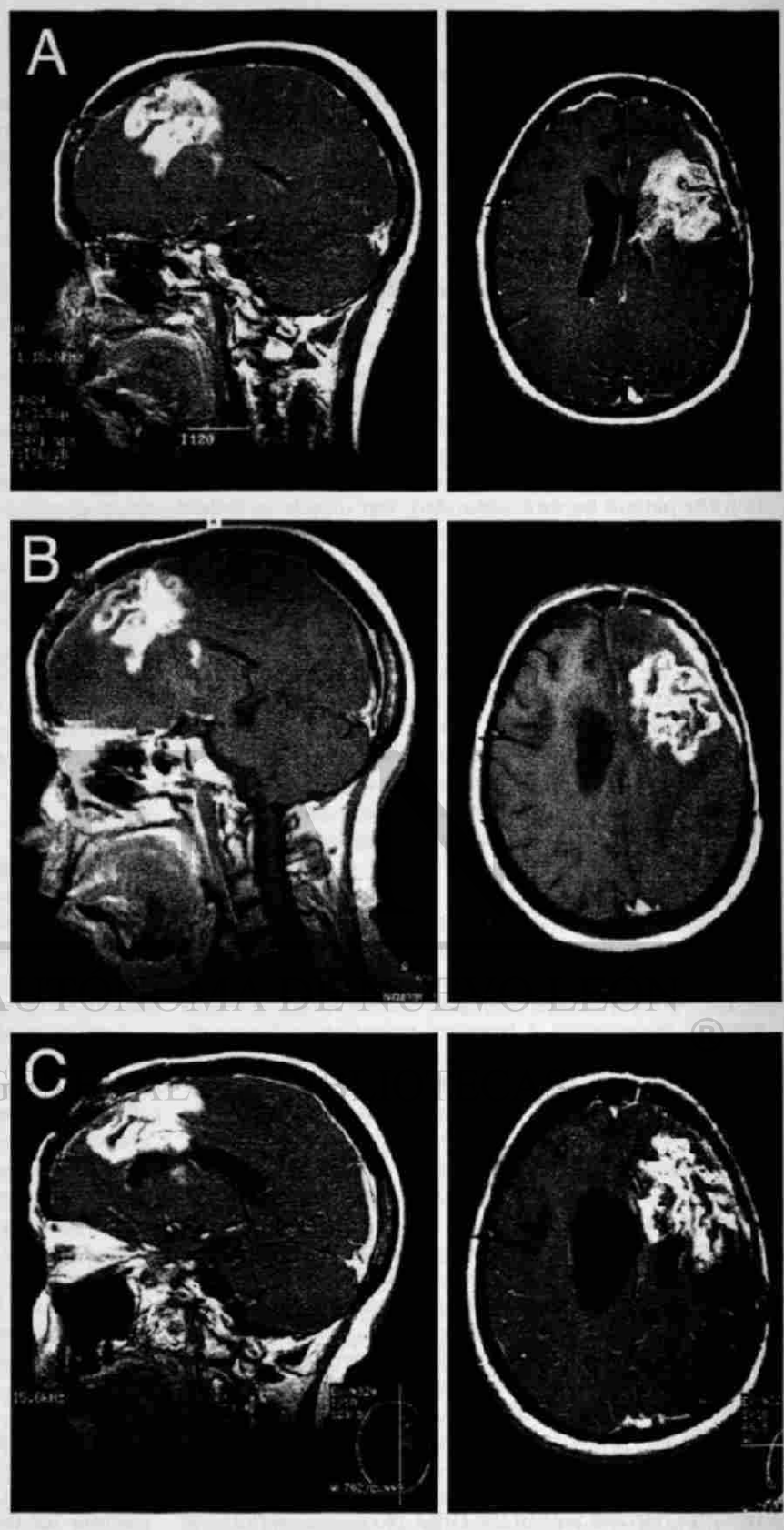

FIG. 1. Forty-two-year-old female (patient 6) with a recurrent left frontal glioblastoma multiforme who received 2×10^{11} VP (1×10^{10} IU) of the Adv.RSVtk vector, followed by intravenous GCV. (A) Pretreatment (1 day before injection of Adv.RSVtk). (B) Two months after vector injection. (C) One year after vector injection. (D) Two years and 2 months after vector injection.

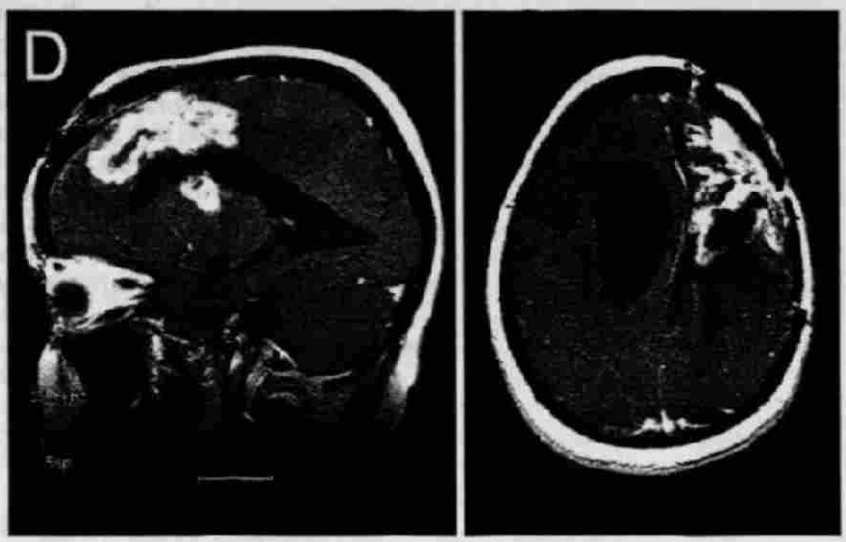

FIG. 1—*Continued*

plemented another Phase I trial of patients with advanced brain tumors in which resection and multiple adenoviral vector injections were being used (44).

We conclude that the form of gene therapy used in our Phase I trial is safe when the vector dose does not exceed 2×10^{11} VP (1×10^{10} IU). There was significant toxicity in patients 8 and 9 who were treated with 2×10^{12} VP. Factors that may have potentiated toxicity in these two patients include older age (age 65), bilateral tumor and hemorrhage in patient 8, and possible intraventricular injection in patient 9.

Toxicity of the nature we observed was not seen in another Phase I brain tumor protocol using an adenoviral vector which is being carried out at the University of Pennsylvania (personal communication, Stephen L. Eck, University of Pennsylvania Medical Center, October 1999). In that trial, patients received two intratumoral injections, 1 week apart, using an adenoviral vector dose of 1×10^{11} pfu. Whether the differences in toxicity observed reflect dissimilarities between the vectors, the doses administered, the patients, or the protocols is currently not known. Although the highest dose we used [2×10^{12} VP (1×10^{11} IU as determined by CPE)] appears similar to that used in the University of Pennsylvania study, comparison of vector doses used in different studies is difficult. Nyberg-Hoffman and associates have demonstrated that the various assays and methodologies used to titer adenoviral vectors can produce markedly different estimates of the number of infectious units in a vector sample (25). In addition, toxicity following injection of an adenoviral vector may relate not only to the infectious dose employed, but also to the physical number of vector particles injected. Standardization of the methods used to determine adenoviral titers is needed in order to compare vector doses and to better assess toxicity.

The toxicity associated with adenoviral vectors appears to be the result of a multifactorial cellular and humoral immune response, which may also play an important role in the destruction of malignant neoplasms. In our Phase I study, neuropathological examination of tumor after vector injection demonstrated marked intratumoral infiltration with inflammatory cells, suggesting that recruitment of an antitumor response of the immune system may play a role in killing tumor cells. Dewey and associates have recently reported long-term inflammation in normal brain in a rat tumor model in which the rats were injected intratumorally with an adenoviral vector (46). We did not observe such severe or widespread inflammatory changes in normal brain tissue in any of the specimens examined.

Three patients in our study, 2 with a GBM (patients 1 and 6) and 1 with an AA (patient 4), survived >25 months, considerably beyond the expected survival for patients with recurrent malignant gliomas. A recent survival study followed a group of 130 patients with glioblastoma multiforme, some of whom underwent chemotherapy (74%) or stereotactic radiation (7%) but not resection, following tumor recurrence (3). These patients had, upon initial diagnosis, undergone resection of the tumor and had received external beam radiation therapy, usually with concomitant chemotherapy. The median survival

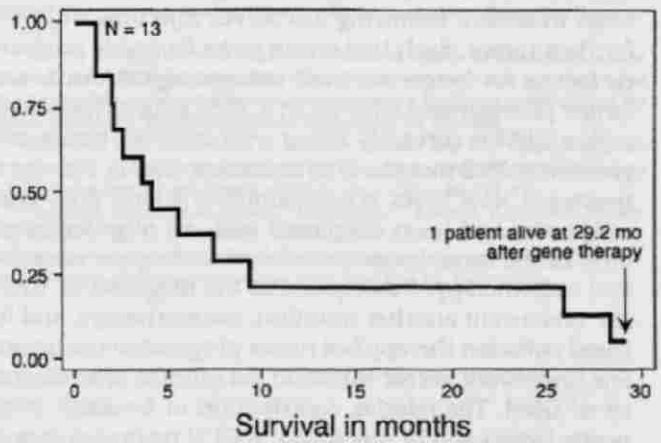

FIG. 2. Patient survival.

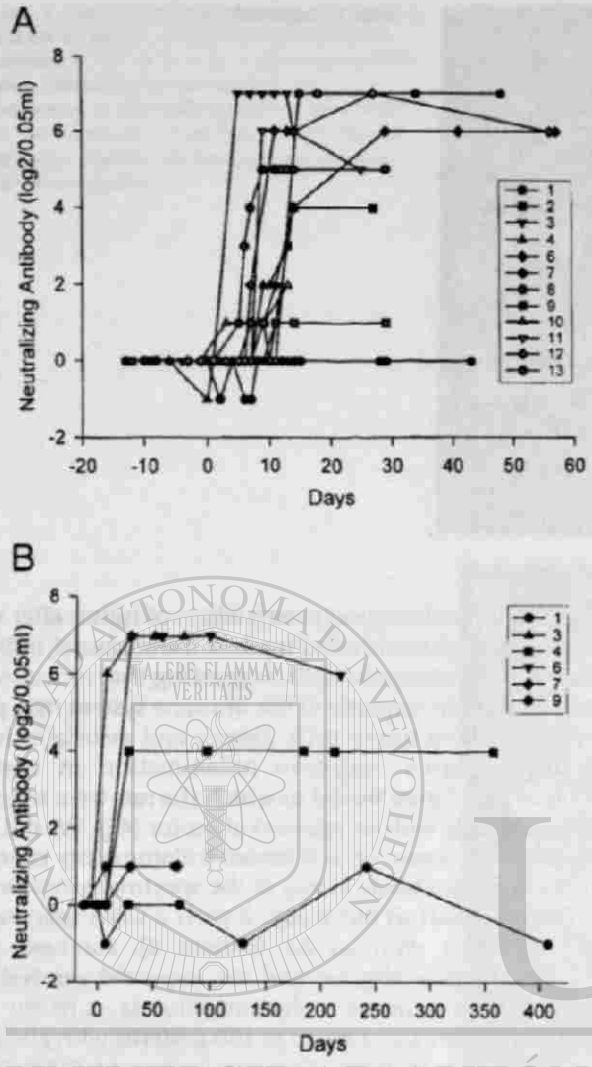

FIG. 3. Adenovirus antibody titers relative to levels immediately before gene therapy (day 0). (A) Titers for 12 patients over the first 2 months after Adv.RSVtk injection. (B) Titers for 6 patients with extended survival after Adv.RSVtk injection.

time after tumor recurrence for these 130 patients was 23 weeks (5.75 months). None of our 3 patients received any other treatment following Adv.RSVtk injection and GCV for their tumor. Each had one or more favorable prognostic factors for longer survival: younger age, AA in 1, and longer pretreatment survival in 2. One patient (patient 6) with a GBM is currently living with minimal tumor progression at 29.2 months after treatment (Fig. 1). Her age at treatment, 41.8 years, is comparatively young. Four years earlier she had been diagnosed with an oligodendroglioma in the same location and had undergone resection and radiotherapy. Subsequent to the diagnosis of GBM, she underwent another resection, chemotherapy, and focused radiation therapy but tumor progression continued. She underwent vector injection 9.8 months after diagnosis of GBM. The relative contribution of favorable prognostic factors and of Adv.RSVtk + GCV treatment cannot be determined in this case.

Current development of gene therapy for malignant brain tumors is being addressed in a number of areas: improving vector efficiency and minimizing immunologic response to the vector (47, 48), exploration of vector constructs utilizing different deletions as well as different promoters, and improvements in the construct or delivery of GCV or similar prodrugs that may extend the bystander effect and reduce GCV-mediated toxicity (14, 15, 49). In addition, the optimal delivery technique must be determined in order to more uniformly distribute the vector throughout the tumor. Possibilities include multiple stereotactic injections, injection of postresection tumor margins, and vector delivery in conjunction with blood–brain barrier disruption. Several centers have recently developed models that improve vector delivery to brain tumors by selectively modifying the blood–brain barrier (50, 51).

Finally, the best results may be obtained when this treatment is combined with other modalities such as radiation therapy, chemotherapy, and other gene therapy approaches. Improved tumor killing has been observed in several cancer models by combining HSV-tk/GCV treatment with radiotherapy (52, 53).

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