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Molecular Lysis of Synovial Lining Cells by *In Vivo* Herpes Simplex Virus-Thymidine Kinase Gene Transfer

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ABSTRACT

Herpes simples virus thymidine kinase (HSV-TK) expression plasmid DNA was injected into the joint space of rabbits with antigen-induced arthritis (AIA). Purified plasmid DNA was able to mediate transfection of synovial lining cells and transient overexpression of HSV-TK in the context of active synovial inflammation. The pharmacodynamic distribution of intraarticular expression plasmid DNA was confined to the joint space. Arthritic rabbits treated with intraarticular expression plasmid DNA followed by intravenous ganciclovir (GCV, 5 mg/kg) twice daily for 3 days showed histologic evidence of synovial lining layer cytolysis when articular tissues were examined 21 days posttreatment. There was also a reduction in joint swelling in the TK-treated knees. No untoward clinical effects were observed in the rabbits and no evidence of cytolytic damage specific to the TK-GCV gene therapy was observed either in the articular cartilage or bone. The application of TK-GCV intraarticular gene therapy using purified expression plasmid DNA for the induction of synovial cytolysis may be applicable to the treatment of human inflammatory arthritis.

OVERVIEW SUMMARY

Previous studies have shown that synovial lining cells are susceptible to *in vivo* transfection using purified expression plasmid DNA. Roessler *et al.* now report on the use of a plasmid that mediates transient overexpression of herpes simplex virus thymidine kinase to transfect synovial lining cells in an animal model of proliferative inflammatory arthritis. After *in vivo* intraarticular transfection using the pNGVL-TK expression plasmid, the animals were treated with intravenous ganciclovir for a period of 3 days. They report that examination of the synovial tissues 21 days after completion of the gene therapy showed evidence of cytolysis that was confined to the synovial lining cells within inflamed synovium. No evidence of cytolysis or necrosis was observed in articular cartilage present within the treated joints. Similar methods to achieve a molecular lysis of the synovial lining layer may have applicability to the treatment of human inflammatory arthritis.

INTRODUCTION

CURRENTLY AVAILABLE systemic therapies for rheumatoid arthritis (RA) do not effectively prevent the progressive loss of cartilage and bone, which leads to joint deformities and functional morbidity. An effective regional treatment that prevented loss of cartilage and periarticular bone erosion would be a cost-effective therapy even if only knee joints could be treated successfully. A treatment directed at the joints of the hands and wrists (which are less amenable to surgical reconstruction) would be even more cost effective. Various methods have been proposed as a means to ablate rheumatoid synovium, including surgery, radiation, and caustic chemicals; however, all of these strategies have suffered from significant side effects or toxicities that have limited the utility of these approaches (Veikko and Laine, 1967; McEwen, 1968; Geens, 1969; Goldie, 1974; Paus, 1990; Deutsch *et al.*, 1993; Cruz-Esteban and Wilke, 1995). From a review of the literature regarding surgical and alternative forms of synovectomy, it is clear that synovectomy has the potential to alter the severity of deformities, or to reduce the rate at which deformities occur over time in a defined rheumatoid joint (Mori, 1985). Unfortunately, surgical synovectomy has significant limitations, including applicability only to large joints, overall cost and complications, particularly ligamentous instability and lost range of motion (Goldie, 1974).

As a safer and perhaps more effective alternative to these methods, we believe that selective synovial lining layer cytolysis can be achieved *in vivo* using a "cellular suicide" gene therapy. The most comprehensively studies of the available cellu-

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lar suicide strategies involves the use of herpes simplex thymidine kinase (TK) as a transgene and the guanosine nucleoside analog ganciclovir (GCV) as the cytotoxic prodrug (subsequently referred to as TK–GCV) (Borelli *et al.*, 1998; Ezzeddine *et al.*, 1991; Culver *et 1992; al.*, 1992; Barba *et al.*, 1993; Oldfield *et al.*, 1993; Ram *et al.*, 1993). Most often the targeted cells are neoplastic in origin; however, other types of proliferating cells have also been used as targets for TK gene transfer (Ohno *et al.*, 1994). Previous studies in our laboratory and others indicates that HSV TK–GCV is the best molecular cytotoxic strategy available, and clearly has the best potential for *in vivo* amplification of the cytotoxic effects from the "bystander effect" (Bi *et al.*, 1993; Freeman *et al.*, 1993; Shewack *et al.*, 1994).

The development of a semitransformed phenotype and proliferation of the synovial lining cells are among the earliest changes that have been characterized pathologically in RA (Yocum *et al.*, 1988; Lafyatis *et al.*, 1989; Gay *et al.*, 1993). It is therefore reasonable to hypothesize that proliferating synoviocytes may also be susceptible to HSV TK–GCV cytotoxicity. We have now performed studies in a rabbit model of arthritis, using TK–GCV gene therapy based on the intraarticular injection of expression plasmid DNA followed by intravenous GCV, a strategy that we have described as a molecular lysis of the synovial lining layer.

MATERIALS AND METHODS

Expression plasmid DNA

The expression plasmids pNGVL-TK and pNGVL-AP were used for *in vivo* transfection studies. These expression plasmids were constructed by the National Gene Vector Laboratory (NGVL) program (University of Michigan Medical Center, Ann Arbor, MI) and both plasmids are based on the pNGVL-1 backbone (Fig. 1). Both plasmids use the cytomegalovirus (CMV) early promoter enhancer to drive transcription, contain the CMV intron A to enhance mRNA processing, and use a β-globin polyadenylation signal sequence. The HSV TK gene is cloned downstream of the CMV promoter in pGNVL-TK. The cDNA for heat-stable human alkaline phosphatase is cloned downstream of the CMV promoter in pNGVL-AP. Large-scale preparations of the expression plasmids were produced by the University of Michigan Vector Core, using a modification of the alkaline lysis method followed by centrifugation over sequential cesium chloride gradients. Endotoxin levels in all of the preparations used *in vivo* were less than 10 endotoxin units/mg DNA. Purified plasmid DNA was resuspended in lactated Ringer's at a concentration of 1 mg/ml prior to use.

Animal studies

All animal experiments were approved by the University Committee for Use and Care of Laboratory Animals and were performed according to American College of Veterinarian Medicine (ACVM) guidelines. New Zealand White rabbits, 2.5 kg in weight and of either sex, were immunized with 4 mg of ovalbumin emulsified in Freund's complete adjuvant by intradermal injection. Animals were boosted 14 days after the initial immunization. Five days following the second immunization, adjuvant arthritis was induced in each knee joint by the intraarticular administration of 5 mg of ovalbumin in 0.5 ml of phosphate-buffered saline (PBS). Using this protocol, the severity of the proliferative arthritis peaks in approximately 6 days and continues for a period of several weeks (Imrie, 1976; Howson *et al.*, 1986; Henderson and Glynn, 1981; Lopez-Garcia *et al.*, 1993). Six days following induction of adjuvant arthritis, the left knee of each animal received an intraarticular injection of pNGVL-TK plasmid DNA in a total volume of 1 ml. The right knee received an equivalent dose of pNGVL-AP plasmid DNA, or an equivalent volume of sterile Ringer's lactate as a control. Twenty-four hours following intraarticular injection the animals received intravenous infusions of ganciclovir (Cytovene; Roche, Nutley, NJ) at a dose of 5 mg/kg. The ganciclovir was administered twice daily for a total of 3 days.

In situ *HSV TK assay*

Samples of synovium, obtained by *en bloc* dissection of the infrapatellar fat pad, were placed into sterile plastic containers, weighed, and then covered with Dulbecco's modified Eagle's medium (DMEM)–10% fetal calf serum (total volume, 5 ml). [8-^{3}H]Ganciclovir (20 μCi/ 1.37 nmol; Moravek Biochemicals, Brea, CA) was added to each sample and the tissue was incubated for a period of 24 hr at 37°C in 5% CO_2. Samples were then washed extensively with sterile PBS until the background radioactivity in the washes was <2.5 cpm/μl. Tissue was then lysed with 0.8 *N* perchloric acid (total volume, 1 ml), and insoluble debris was removed by centrifugation. The supernatants were titrated to pH 7.0 with potassium hydroxide and insoluble debris was removed by centrifugation. Triplicate aliquots of the neutralized supernatants were assayed for [8-^{3}H]ganciclovir, using a liquid scintillation counter, with results expressed as disintegrations per minute per milligram of synovial tissue per 24 hours.

Polymerase chain reaction

Samples of major organ systems were obtained at the time of necropsy. Tissues were snap frozen using liquid nitrogen and stored at −80°C until use. Whole cellular DNA was isolated from the frozen tissues using a QiAmp kit (Qiagen, Chatsworth, CA) according to the manufacturer protocol. One microgram of total DNA isolated from each of the tissues was then subjected to polymerae chain reaction (PCR) amplification, using sense and antisense oligonucleotide primers specific for pNGVL sequences flanking the herpes simplex thymidine kinase gene. The sequence of the 5′ forward primer was 5′ TCCATGGGTCTTTTCTGCAG 3′ and the sequence of the 3′ reverse primer was 5′ CAGATGCTCAAGGGGCTTCA 3′. PCR conditions were as follows: 94°C for 1 min, 65°C for 30 sec, and 72°C for 2 min for a total of 35 cycles, and all PCRs were performed in triplicate. Aliquots of the PCR product were electrophoresed in 1% agarose gels containing ethidium bromide and photographed. The sensitivity of the visually scored PCR assay was determined to be 10 plasmid copies per microgram of genomic DNA.

Histology

Animals were euthanized and necropsies were performed. Representative samples of all major organ systems were ob-

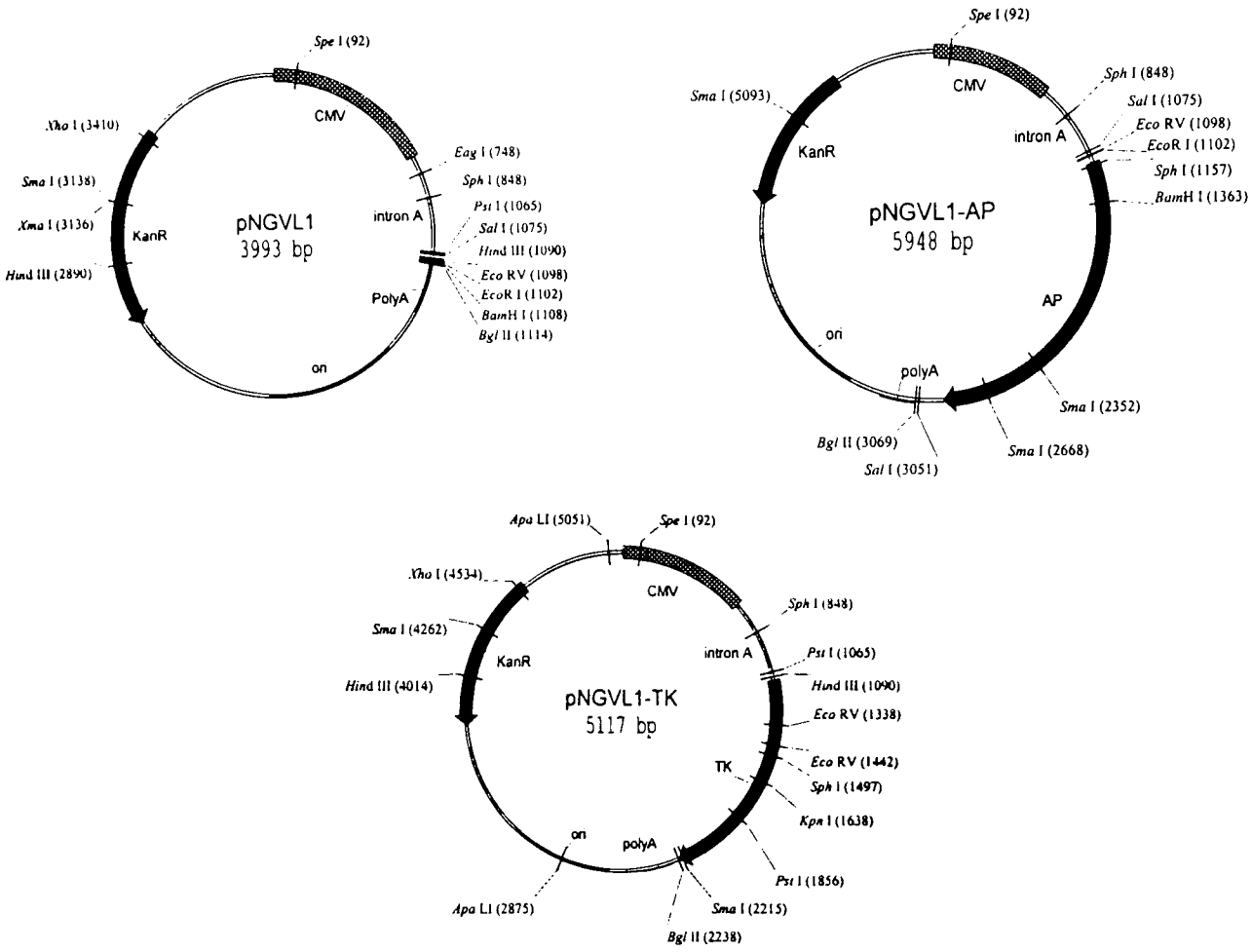

FIG. 1. Maps of expression plasmids pNGVL1, pNGVL-TK, and pNGVL-AP. pNGVL-1 is a pUC-based expression plasmid that uses the CMV early promoter enhancer to drive transcription, contains the CMV intron A to enhance mRNA processing, and uses a β-globin polyadenylation signal sequence. The HSV TK gene is cloned downstream of the CMV promoter in pNGVL-TK. The cDNA for heat-stable human alkaline phosphatase is cloned downstream of the CMV promoter in pNGVL-AP.

tained from each animal and were immersion fixed in 10% buffered formalin. Tissues were embedded in paraffin and sections (6 μm) were placed onto glass slides and stained with hematoxyin and eosin. Sections were examined by light microscopy for any evidence of pathologic changes.

Knees were isolated *en bloc*, using sharp dissection, and then immersion fixed in 10% buffered formalin, decalcified in formic acid, divided in half along a sagittal plane and embedded in paraffin. Serial sections (6 μm) were obtained from each block, placed onto glass slides, and stained with hematoxylin and eosin.

The sections were then examined in blinded fashion (M.B.) and the degree of synovial inflammation was determined using the criteria established by Paus *et al.* (Paus, 1996). The extent of synovial lining layer cytolysis was also determined.

In vitro *bystander effect*

RA synoviocytes were transfected with pNGVL-TK in order to achieve a transduction efficiency of approximately 5%. The transfected cells were then mixed with untransfected synoviocytes at ratios of 1:0, 1:1, 1:5, and 1:10. The cells were plated a density of 10^4 cells/well into a 96-well plate. Mock transfectants were used as negative controls, and an NIH 3T3 TK retroviral producer cell line was used as a positive control. Cells were allowed to adhere to the plate for 24 hr and were then exposed to medium containing GCV at a concentration of 10 μg/ml for a period of 24 hr. The rate of proliferation in each group of cells was determined in triplicate at serial time points (3, 8, and 11 days) using a tetrazolinium-based assay (CellTiter96; Promega, Madison, WI).

RESULTS

Intraarticular administration of pNGVL-TK mediates in vivo *transfection and expression of transgenic HSV TK*

Initial experiments were designed to determine if acutely inflamed synovium would be susceptible to *in vivo* transfection using naked plasmid DNA. Highly purified preparations of

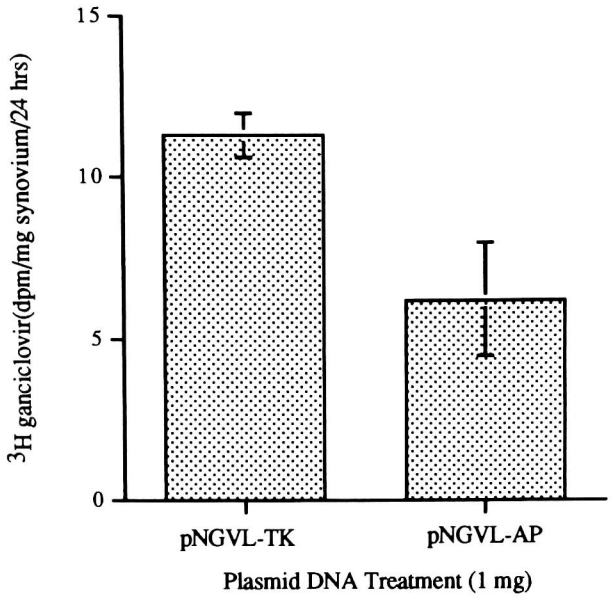

FIG. 2. *In situ* incorporation of ganciclovir by arthritic synovium transfected *in vivo* with pNGVL-TK. Triplicate aliquots of neutralized synovial tissue supernatants were assayed for [8-^{3}H]ganciclovir, using a liquid scintillation counter, with results expressed as disintegrations per minute per milligram of synovial tissue per 24 hours. On the basis of the specific activity of the [8-^{3}H]ganciclovir used for these studies we calculated that the synovial tissue treated with pNGVL-TK incoporated 7.1×10^{-17} mol of ganciclovir per milligram of synovium per 24 hours. Controls treated with pNGVL-AP incorporated 3.9×10^{-17} mol of ganciclovir per milligram of synovium per 24 hours. These differences were statistically significant by analysis using Student's t test ($p < 0.01$). Each histogram represents mean values ($n = 4$).

pNGVL-TK were made using fast protein liquid chromatography (FPLC). Synovial pannus tissue was transfected *in vivo* by intraarticular administration of pNGVL-TK expression plasmid DNA, then isolated and cultured *in situ* with tritiated ganciclovir. We hypothesized that the radiolabeled ganciclovir present in the tissue culture medium would freely pass into the cytoplasm of all of the synovial lining cells and over a period of several hours would reach steady state levels (Mahony *et al.*, 1988). A small amount of this ganciclovir was expected to be converted by endogenous TK into mono-, di-, and triphosphate metabolites that would be retained within all of the cells. However, over the same period of time transfectants expressing transgenic HSV TK would convert a much larger percentage of the radiolabeled ganciclovir into mono-, di-, and triphosphate metabolites that would be retained within transfected synovial lining cells (Mesnil *et al.*, 1996). We predicted that the total amount of ganciclovir and ganciclovir metabolites present in the total population of cells analyzed (both transfectants and normal cells) would be directly proportional to the total amount of biologically active TK. Differences between tissues treated with pNGVL-TK and a control plasmid would indicate specific expression of transgenic HSV TK. The results of this study are presented in Fig. 2.

Pharmacokinetic distribution of pNGVL-TK plasmid DNA following intraarticular injection into rabbits with antigen-induced arthritis

The purpose of this experiment was to ascertain the pharmacokinetic distribution of plasmid DNA following a single intraarticular injection into an actively inflamed knee joint. Six days after induction of adjuvant arthritis, plasmid DNA (1 mg of pNGVL-TK) was injected into the left knee of the rabbits ($n = 4$; two females and two males). Twenty-four hours after

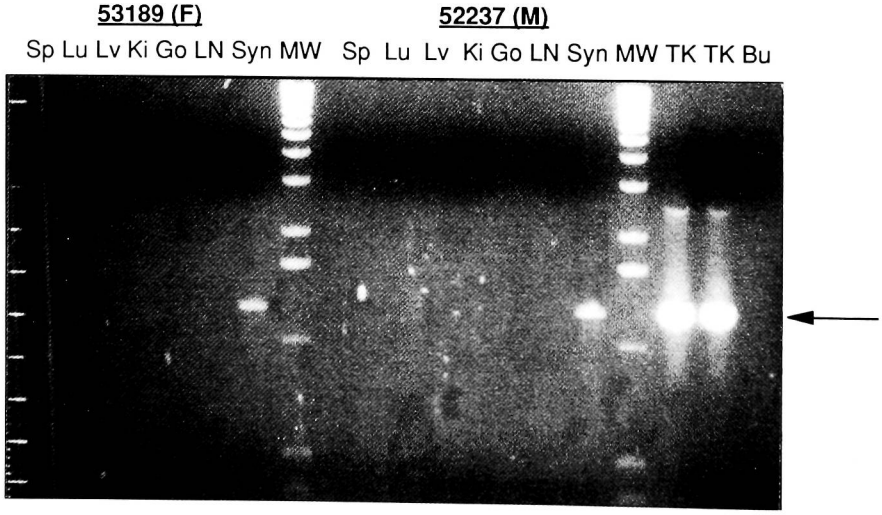

FIG. 3. Photograph of an agarose gel stained with ethidium bromide following electrophoresis of PCR products from two of four rabbits (rabbits 53189, female and 52237, male) treated with intraarticular pNGVL-TK. A 1.15-kbp band (arrow) corresponding to the herpes simplex thymidine kinase gene is readily visualized in PCR-amplified samples of total DNA (1 μg) obtained from the synovium of animals 24 hr after intraarticular injection of pNGVL-TK DNA. No bands were observed in DNA obtained from the other organs examined. A 1.15-kbp band was also observed in PCR products from duplicate samples containing purified pNGVL-TK plasmid DNA (TK). No bands were observed in samples of buffer alone (Bu). Sp, Spleen; Lu, lung; Lv, liver; Ki, kidney; Go, gonads; LN, inguinal lymph nodes; Syn, synovium; MW, molecular weight markers; TK, pNGVL-TK plasmid DNA; Bu, buffer alone.

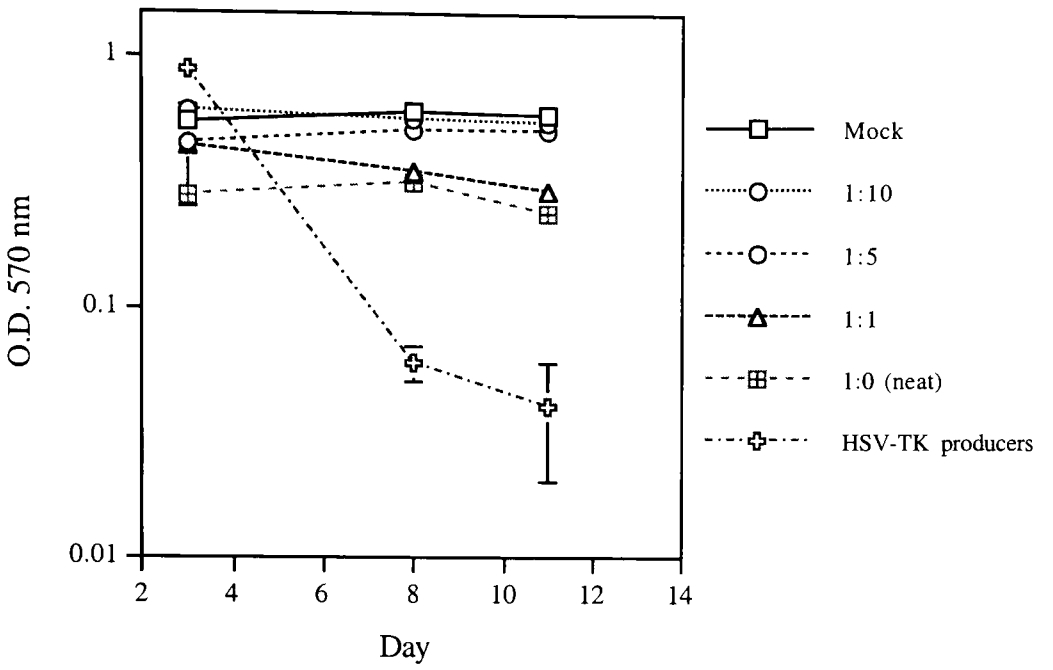

FIG. 4. Antiproliferative effects of transgenic HSV TK–ganciclovir treatment in rheumatoid synoviocytes *in vitro* appears to be mediated via a bystander effect. Rheumatoid synovial fibroblasts were transfected with pNGVL-TK, using calcium phosphate, to an estimated transfection efficiency of 5%. Twenty-four hours posttransfection the entire population of cells was recovered by trypsinization and then mixed with untransfected synoviocytes at ratios of 1:0, 1:1, 1:5, and 1:10, and plated at a density of 10^4 cells per well into 96-well plates. Mock transfectants were used as negative controls and an NIH 3T3 TK retroviral producer line was used as a positive control. Cells were allowed to adhere to the plates overnight and then were exposed continuously to complete medium containing ganciclovir (10 μg/ml), for a period of 24 hr. The rate of proliferation in each group of cells was determined in triplicate at serial time points (3, 8, and 11 days) using a tetrazolium-based assay (CellTiter96; Promega). Results are expressed as optical density (OD) at 570 nm. Error bars indicate SEM.

intraarticular injection the rabbits were euthanized and necropsied. Samples were obtained from multiple organ systems; lung, liver, kidney, spleen, inguinal lymph nodes (ipsilateral to the injection), gonads, and synovium and were subjected to DNA PCR amplification. Representative results are depicted in Fig. 3.

As expected, a 1.15-kbp PCR fragment of the herpes simplex thymidine kinase gene was readily amplified from genomic DNA isolated from treated synovium in all four animals. None of the other tissues obtained from the animals had evidence of an amplifiable template of herpes simplex thymidine kinase gene present within organ systems obtained 24 hr after a single intraarticular dose. These results suggest that following intraarticular injection, plasmid DNA does not readily escape from the confines of the joint space even in the context of active synovitis.

Bystander effect of rheumatoid arthritis synoviocytes transfected with pNGVL-TK in vitro

With a transduction efficiency of approximately 5% (similar to that expected following *in vivo* intraarticular administration of plasmid DNA), there was a significant antiproliferative effect of the entire synoviocyte population for all groups of cocultured cells. This suggests that even if only a small number of synoviocytes express the HSV TK transgene, widespread killing of the entire cell population (both transfected and untransfected) was observed; positive evidence of the bystander effect. Thus with even this low rate of transfection, the biological effect of cytotoxicity was substantial. The results are shown in Fig. 4.

Intraarticular administration of pNGVL-TK followed by intravenous administration of ganciclovir induces cytolysis of transfected synovial lining layer in vivo

We next performed a series of *in vivo* experiments designed to determine the effects of plasmid-mediated transgemic expression of HSV TK in synovial pannus tissue followed by the parenteral administration of ganciclovir twice daily for 3 days. Rabbits were euthanized 21 days after administration of the pNGVL-TK plasmid and the knee joints were isolated *en bloc*, fixed, and sectioned. Serial sagittal sections were then examined and the amount of inflammation and synovial lining layer cytolysis was quantified. The results of a representative experiment are presented in Fig. 5. Qualitatively, knees treated with pNGVL-TK and GCV (n = 10) showed histologic evidence of synovial lining layer cytolysis while control knees treated with pNGVL-AP and GCV (n = 6) showed no evidence of treatment-related synovial lining layer cytolysis. Additional controls that received no treatment (n = 4) or treatment with GCV alone (n = 2), or pNGVL-TK alone (n = 2), also showed no evidence

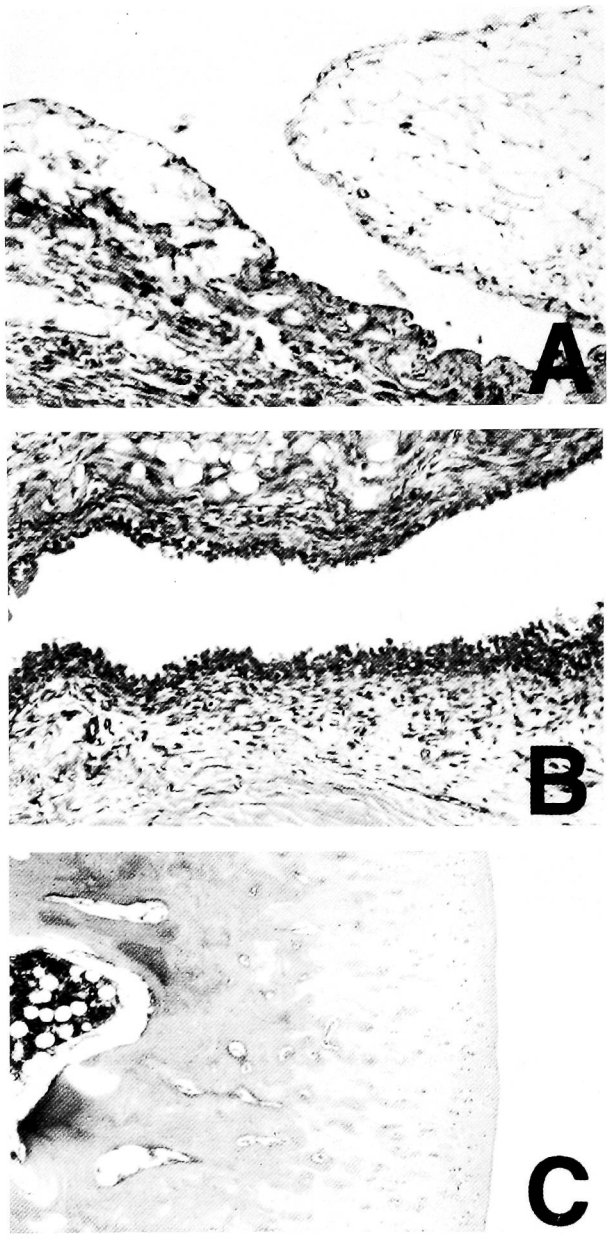

FIG. 5. Intraarticular administration of pNGVL-TK followed by intravenous administration of ganciclovir induces cytolysis of the transfected synovial lining layer *in vivo*. (**A**) Marked loss of the synovial lining cells within a knee treated with pNGVL-TK, with evidence of reduced inflammation. (**B**) The contralateral control knee, which showed evidence of continuing synovial hyperplasia, subintimal neovascularization, and infiltration of chronic inflammatory cells. (**C**) Normal bone and cartilage from the TK-treated knee shown in (**A**). Original magnification: (**A** and **B**) ×115; (**C**) ×57.5.

of treatment-related synovial lining layer cytolysis. A representative quantitative assessment of the degree of synovial inflammation and synovial lining layer cytolysis in four animals treated with pNGVL-TK and GCV is shown in Table 1.

Intraarticular administration of pNGVL-TK followed by intravenous ganciclovir reduces joint swelling in TK-treated knees

We next performed a series of knee measurements to determine whether there was a clinical effect related to the aministration of pNGVL-TK followed by intravenous ganciclovir. In this experiment, which included four rabbits, the experimental procedure was as previously described except that a chronic arthritis was maintained following initial induction by means of repeat intraarticular (IA) injections of 0.25 mg of ovalbumin in 0.25 ml of sterile PBS into each knee. The maintenance IA ovalbumin was administered on days 15, 47, and 61 following initial arthritis induction on day 1. Intraarticular plasmid DNA was administered on day 75. Using standard calipers, the mean mediolateral diameter of each knee of four rabbits was measured on days 15, 47, 61, 75, 92, 95, 117, 126, and 138. Knees treated with pNGVL-TK followed by intravenous ganciclovir showed a sharp reduction in knee swelling compared with the untreated knees in the period immediately following plasmid administration and until day 96, during which time the untreated knees continued to increase in size. The differences between the TK-treated knees and the untreated knees approached statistical significance at day 96 ($p = 0.12$, Student's t test). After this point and up to day 117, the rate of improvement in arthritis in TK-treated and untreated knees paralleled each other. This was followed by another period of rapid improvement in joint swelling from day 117 to day 138 in the TK-treated knees ($p = 0.15$, Student's t test). TK-treated knees were smaller than the untreated knees at all time points. The results are shown in Fig. 6.

DISCUSSION

It is clear that many types of inflammatory arthritis in humans, as well as animal models of inflammatory arthritis, are

TABLE 1. QUANTITATIVE ASSESSMENT OF SYNOVIAL INFLAMMATION AND SYNOVIAL LINING LAYER CYTOLYSIS 21 DAYS FOLLOWING TREATMENT WITH INTRAARTICULAR pNGVL-TK FOLLOWED BY PARENTERAL GANCICLOVIR[a]

Rabbit No.	*Left knee (treated with pNGVL-TK and GCV)*		*Right knee (untreated)*	
	Inflammation	*Cytolysis*	*Inflammation*	*Cytolysis*
1	1	3	2	0
2	1	2	3	0
3	0	3	1	0
4	1	2	2	0

[a]Serial sagittal sections from each knee joint were stained with hematoxylin and eosin and then quantitatively examined for the degree of synovial inflammation and synovial cytotoxicity. The findings were visually estimated on the basis of the extent of changes seen. The following grades were used: Grade 0, no abnormality observed; grade 1, <30% of tissue showing abnormality; grade 2, 30–74% of tissue showing abnormality; grade 3, >75% of tissue showing abnormality.

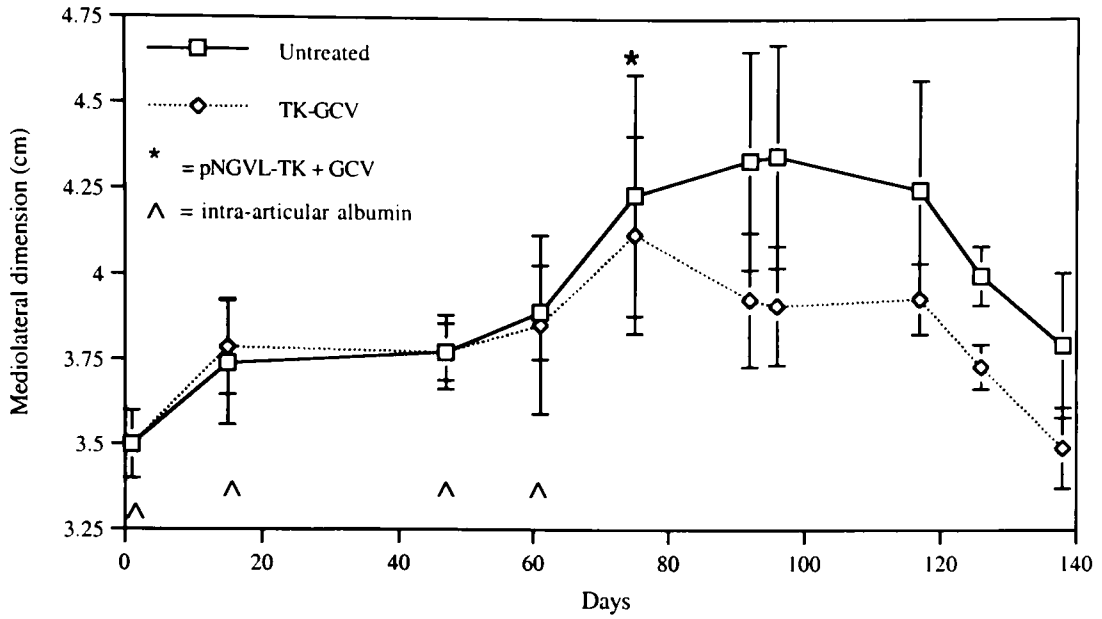

FIG. 6. Serial measurements of mean mediolateral knee diameter in four rabbits with bilateral chronic inflammatory knee arthritis treated with intraarticular pNGVL-TK followed by intravenous ganciclovir. Knee measurements were obtained on days 1, 15, 47, 61, 75, 92, 95, 117, 126, and 138 following induction of arthritis. Intraarticular HSV-TK (1 mg/ml) was administered into the left knee of each rabbit on day 75; the right knee was untreated. Intravenous ganciclovir (5 mg/kg) was administered twice daily to each rabbit on the three successive days following administration of HSV-TK. In the period immediately following plasmid administration, TK-treated knees showed a sharp reduction in knee swelling compared with the untreated knees. The p value for the difference in knee swelling between TK-treated and untreated knees on days 96 and 75, and on days 117 and day 75, approached statistical significance (0.12 and 0.15, respectively). The rate of improvement in knee arthritis in the TK-treated knees was greater than in the untreated knees at all times.

associated with the proliferation of synovial fibroblasts. A major pathological feature of RA is an uncontrolled proliferation of synoviocytes that is associated with bone erosion; synovial hyperproliferation is important in both the acute and chronic phase of the disease. Synovial fibroblasts also participate directly in the cellular processes that result in destruction of cartilage and bone and the development of a state of chronic immune activation (McCachren, 1991; Harris, 1992; Gay *et al.*, 1993; Kinne *et al.*, 1995). It is therefore a rational starting point to hypothesize that proliferating synoviocytes are appropriate targets for a TK–GCV therapeutic strategy. The goal of this study was to analyze the effects of a TK–GCV gene therapy in an animal model of arthritis.

It would be expected that the results of a TK–GCV-based strategy would be related to the proliferative index of the target lesion. In patients enrolled in chronic RA immunohistochemical staining studies using the proliferation antigen Ki 67, the rates of synovial proliferation were estimated to be on the order of 5% (R.W. Kinne, personal communication). In the antigen-induced arthritis model the rate of synovial proliferation 6 days after intraarticular challenge with ovalbumin is also approximately 5% (Henderson *et al.*, 1982). We therefore chose this time point for the administration of intraarticular plasmid. Although the rate of synovial proliferation in the antigen-induced model is maximal between days 3 and 7, rates of proliferation continue to be elevated for up to 84 days following challenge (Henderson *et al.*, 1982).

In addition to the proliferation of synovial fibroblasts, rheumatoid synovitis is characterized by hyperplasia of synovial monocytes and infiltration of activated (proliferating) T and B lymphocytes. This cellular milieu suggests that many of the cells that contribute to the pathology of RA may be susceptible to TK–GCV-induced cytotoxicity either directly (as in the case of synovial fibroblasts) or indirectly through the well-described "bystander" effects (Bi *et al.*, 1993; Freeman *et al.*, 1993; Shewack *et al.*, 1994). Bystander effects are thought to be mediated primarily through the transfer of ganciclovir triphosphate into neighboring cells through gap junctions of intercellular communication (GJIC) (Harris, 1992). Preliminary studies in our laboratory using immunohistochemical staining methods suggest that rheumatoid synovial fibroblasts express connexin 43, a component protein of GJIC (data not shown). Our *in vitro* data also show that the bystander effect is important in synovial cell cytolysis following transfection of synovial tissue with HSV TK followed by ganciclovir. This suggests that a similar mechanism may apply *in vivo*.

Several groups have now reported that synovial lining cells are susceptible to transient transfection *in vivo* following the intraarticular administration of expression plasmid DNA (Yovandlich *et al.*, 1995; Nita *et al.*, 1996). This process of spontaneous transfection occurs via both nonspecific endocytosis and pinocytosis by the synovial lining cells (Yovandlich *et al.*, 1995). There was, however, considerable variability in the level of *lacZ* expression within the synovium in these stud-

ies. Any estimate of efficiency of gene transfer using *lacZ* is approximate owing to the nature of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining and the presence of background staining in resident macrophages. We believe that *in vivo* transfection efficiencies in the AIA model are less than 1% of the total synovial cell population, using *lacZ* as a reporter transgene and visual assessment of X-Gal-stained sections (data not shown).

We hypothesized that this biological property of synovium could be exploited by using a well-described cellular suicide gene therapy strategy, HSV TK–GCV, in the context of active proliferative synovitis by simply administering an HSV TK expression plasmid into the joint space. The use of naked plasmid DNA has several advantages over the use of recombinant viral vectors: the absence of viral coat proteins, exclusive transcription of the desired transgene, and ease of manufacture. The use of naked DNA also obviates the use of lipid excipients, which may have undesirable side effects including focal cellular toxicity and the activation of complement. These present studies show that abnormal synovium is also susceptible to *in vivo* transfection using purified TK expression plasmid DNA. Purified expression plasmid DNA is a colloidal material that has physicochemical properties similar to those of hyaluron and other high molecular weight proteins that are normal components of synovial fluid, and thus our results are not unexpected since rheumatoid synovium may have enhanced phagocytic properties compared with normal synovium (Krakauer and Zurier, 1980).

Ganciclovir is a nucleoside analog that can be phosphorylated to a toxic metabolite by thymidine kinase. Because synovium lacks a basement membrane, small molecules present in synovial capillaries readily pass from serum into synovium and synovial fluid. Uric acid is the classic example of a nucleoside metabolite that equilibrates between serum and synovium (Simkin and Pizzorno, 1974). Intravenously administered ganciclovir also passes freely into synovium and synovial fluid and can be phosphorylated by HSV TK into ganciclovir triphosphate. In addition to the transfer of ganciclovir triphosphate to adjacent synovial cells via gap junctions, the anatomic structure and physiologic function of synovium suggest that ganciclovir triphosphate may pass from dying cells diffusely throughout the joint space, further potentiating the bystander effect. This is relevant in RA, as studies have demonstrated apoptosis in synoval fibroblasts of the subintima (Firestein *et al.*, 1995). The explanation for the low population of apoptotic cells in rheumatoid synovium is that the adjacent synovial macrophages rapidly scavenge the apototic synovial cells (Nakajima *et al.*, 1995).

This study demonstrates that intraarticular HSV TK followed by intravenous ganciclovir has both clinical and histologic effects on antigen-induced arthritis. The cytotoxic effects associated with the TK–GCV gene therapy within the joint space appear to be confined to the synovial lining cells. Additional animal studes will be required to assess the long-term consequences of intraarticular TK–GCV gene therapy. Our short-term results suggest that the intraarticular TK–GCV therapy is clinically well tolerated by large animals and is not associated with pathologic effects in articular cartilage or bone. TK–GCV induces widespread but selective synovial lining layer cytotoxicity, reduces synovial inflammation, and decreased joint swelling.

CONCLUSION

The results of this study suggest that the intra-articular injection of purified TK expression plasmid DNA combined with parenteral ganciclovir can be used to mediate synovial lining layer cytolysis *in vivo* in the context of active inflammatory arthritis. The experimental gene therapy reduces joint swelling and does not appear to be associated with evidence of systemic toxicity or toxic effects in cartilage or bone within the treated joint. Further study of intra-articular TK-GCV gene therapy for the induction of molecular synovectomy may yield regimens that may be applicable to the treatment of humans with active rheumatoid arthritis.

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