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RETROVIRAL SUICIDE VECTOR DOES NOT INHIBIT NEOINTIMAL GROWTH IN A PORCINE CORONARY MODEL OF RESTENOSIS

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Summary: We attempted to attenuate neointimal formation following vascular injury using a retroviral suicide vector. Epicardial coronary arteries of adult miniature swine were injured by deployment of oversized tantalum stents. One week later, injured segments were exposed to packaged retroviral constructs with or without the herpes simplex virus thymidine kinase gene. Ganciclovir treatments were initiated 48 hours later in both control and experimental swine and continued for two weeks. Four weeks following vascular injury, both control and experimental arteries were harvested and histologically prepared for image analysis. Despite adequate marker gene expression, there was no significant difference in the neointimal area or neointimal/media ratio between control and experimental groups. While the HSVtk ganciclovir system attenuates cell proliferation in other systems, retroviral vector targeting of vascular smooth muscle cells for elimination may be too inefficient to prevent restenosis following angioplasty.

Percutaneous transluminal coronary angioplasty (PTCA) has gained widespread approval as a treatment for coronary artery disease; the number of procedures may soon exceed 500,000/year (1). Initial success rates have improved to approximately 90% in the last decade (2, 3). Nevertheless, the clinical utility of this procedure is limited by restenosis in approximately 33% to 50% of patients (4). Furthermore, neither new devices (5) nor a variety of pharmaceutical agents employed to limit smooth muscle proliferation (6) have proven successful in reducing the incidence of PTCA-restenosis. Alternate approaches including gene therapy are under investigation.

The aggressive nature of vascular smooth muscle cell (VSMC) proliferation leading to atherosclerosis and restenosis has led some investigators to compare this process with the cellular proliferation seen in some forms of non-metastatic cancer (7). Numerous animal studies have produced evidence that the early response to vascular injury (and possibly angioplasty) is characterized by a population of dedifferentiated (synthetic phenotype) medial VSMC that will

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eventually comprise the restenotic lesion. These cells proliferate rapidly, while normal medial VSMC replicate at a rate of less than 0.1%/day (8). These observations led us to reason that gene therapies aimed at eliminating transformed, but not normal cells might be useful in attenuating restenosis following angioplasty. One such strategy has been to modify cells to express the herpes simplex virus thymidine kinase (HSVtk) gene. These cells then become vulnerable to treatment with the antiviral agent ganciclovir, while normal cells are unaffected (9, 10). This strategy can be used *in vivo* to specifically target dividing cells by use of retroviral vectors, which are unable to transfer genes into non dividing tissue. Indeed, Plautz et al. have demonstrated successful treatment of adenocarcinoma in mice with such a system, while non dividing cells in rabbit arteries were unaffected (11). Therefore, we have applied such a system to a model of restenosis involving oversized stent injury to swine coronary arteries. Unfortunately, the suicide vector approach did not reduce neointimal formation in this model of vascular injury.

MATERIALS AND METHODS

Construction of retroviral vectors : The LNCX construct (L = LTR, N = neomycin phosphotransferase gene, C = cytomegalovirus promoter, X = cloning site) was digested with the restriction enzyme Hpa I, while the LNSX (S = simian virus 40 (SV40) promoter) construct was digested with Hind III. Both were end-filled and treated with calf intestinal phosphatase (CIP). The pXP2 plasmid (12) was digested with Hind III/PstI to liberate the luciferase cDNA. This digest was end-filled with T_4 DNA polymerase and electrophoresed on a 1% agarose gel to recover the 3 kb luciferase fragment. The luciferase cDNA was then ligated to restricted, end-filled LNSX and LNCX. The HSV-TK gene was removed from pBSHSV-TK (13) with Bgl II/Nco I and Klenow end-filled. It was then ligated into Hind III-digested LNSX (S=simian virus 40 (SV40) promoter). The orientation of LNSTK was determined using Stu I/Sac I. The LNSX construct was used as a control.

Retroviral producer cell lines: A two-step process was used to generate the retroviral producer cells in a manner similar to that used by Dr. Dusty Miller (14). Essentially, the ecotropic cell line, Psi2, was first transfected with the LNCLuc, LNSTK or LNSX construct. The virus harvested from these cells was used to infect the PA317 amphotropic cell line. This circuitous method was employed because there is some evidence to suggest that producer cells generate virus at a higher titer if the DNA construct is introduced by infection rather than transfection. Presumably, this is because the DNA integrates into transcriptionally active domains and is expressed at higher levels. Each of the vectors were used at titers of 10^6 cfu/ml or greater (as determined by colony assays on NIH-3T3 cells). To assess the effectiveness of the suicide vector strategy *in vitro*, producer cells were plated at an initial density of 5 x 10^5/60 mm culture dish and grown for two days. Cells were then treated with ganciclovir twice per day at concentrations from 7.8 to 780 nM for six days. At day six, the cells which remained attached to the culture dishes were stained with trypan blue for viability, harvested and counted.

Vascular injury and gene transfer: Castrated adult male miniature swine (25-30 kg; S&S Farms; Ranchita, CA) were allowed several days acclimatization before experimentation, and were fed a normolipemic diet (Purina diet #5081 - Lab Mini-pig grower chow) for the duration of the study with the exception of the day before and day of surgery. Water was also withheld for ~16 hours before surgery. On the morning of surgery, animals were sedated with a cocktail of ketamine-acepromazine (10/0.5 mg/kg, im). A low dose of atropine (~0.05 mg/kg, i.m.) was administered simultaneously to reduce salivary secretions. Anesthesia was induced with thiopental (~10 mg/kg iv via ear vein) and then maintained with Isoflurane (2-4%; balance O_2) following intubation for the duration of the procedure. Body temperature was maintained with a water filled heating pad. All animals received prophylactic anti-microbial therapy consisting of iv Septra (160 mg Trimethoprim & 800 mg Sulfamethoxazole diluted in 50 ml saline) and Bicillin (600,000 units of Penicillin G benzathine and Penicillin G procaine im;

Wyeth) prior to surgery. Using sterile technique, the right or left femoral artery was isolated and temporarily occluded with vascular loops. An 8 French percutaneous transluminal coronary angioplasty sheath was inserted into the artery, and the animals were heparinized (~200 Units/kg, iv.). Subsequently, an appropriate PTCA guide catheter was positioned at the coronary ostium using fluoroscopic guidance and intravascular contrast (Optiray 320; Mallinckrodt, St. Louis, MO). Bretylium was administered prophylactically as an antiarrhythmic at a dose of 5 mg/kg, iv. Following administration of ~250 ml of Dextran-40 to impair platelet aggregation and ~200 μg of nitroglycerin directly into the coronary artery to prevent damage induced spasm, we passed a PTCA balloon with a tantalum stent (3.0 mm diameter) over a 0.014" guide wire into the left anterior descending, circumflex or right coronary artery. The selected four cm. section (stent length = 18 mm + an area ~1 cm proximal and distal for later balloon deployment) was free of major side branches. Vascular injury was produced by inflating the balloon (~6 -8 ATM), which deployed the wire stent while over stretching the artery. Following balloon deflation, swine received an additional 200 Unit/kg dose of heparin. The catheter was withdrawn, the femoral artery ligated proximal and distal to the point of sheath insertion, and the skin incision sutured with 3-0 silk. The vessel entry site was sufficiently distal to allow abundant collateral flow to the leg from other vessels. Swine received an additional 250 ml of Dextran-40 just before recovery from anesthesia. Following recovery from anesthesia, all animals received approximately 0.3 mg of the opioid analgesic Buprenex (Buprenorphine; Norwich Eaton Pharmaceuticals, NY) for relief of post-operative pain. Flavored aspirin (81 mg/day) was administered orally with food two days prior to injury until sacrifice to reduce the incidence of thrombosis following stent deployment.

Gene transfers were performed one week following stent deployment, during the period of rapid VSMC proliferation (15, 16). Swine were sedated, anesthetized, and instrumented with the appropriate sheath and guide catheter and medicated with heparin, nitroglycerin and bretylium as described above, except that the opposite femoral artery was used for vascular access. A double-balloon perfusion device was deployed into the previously injured coronary artery (USCI-Bard; Figure 1). The design of this device allows coronary flow distal to the isolated segment via perforations in the catheter wall. The tantalum stent served as a precise marker for the site of vascular injury. Following balloon inflation, the isolated segment was flushed with sterile culture media (DME high glucose, no serum; Gibco BRL) to remove blood in the lumen. The vascular segment was then exposed to ~4 X 10^7 pfu (plaque forming units) of a retroviral vector solution composed of the luciferase, HSVtk or control construct in 8 μg/ml polybrene (hexadimethrine bromide, Sigma) via the infusion port (Figure 1). The perfusion catheter was periodically flushed with heparinized saline to maintain distal coronary flow. The injured site remained exposed to the slowly infused (~2 ml/min) retroviral vector for ~ 20 min (previously shown to be adequate for DNA transfer - 21). Supplemental doses of bretylium and additional saline were given if necessary to prevent arrhythmias and maintain mean arterial pressure (MAP) above 80 mm Hg. The balloons were then deflated and the catheter removed. All materials in contact with the retroviral vector were disinfected and/or discarded. The surgical wounds were closed and the animal received supplemental heparin, antimicrobial and analgesic therapy as described above. The first group of swine (n=6) received the luciferase construct within the retroviral vector. Subsequent animals received the retroviral vector with HSVtk or control construct. The identity of the construct was coded and known only to the molecular biologist (JLC) to prevent bias in surgical procedures, ganciclovir administration or image

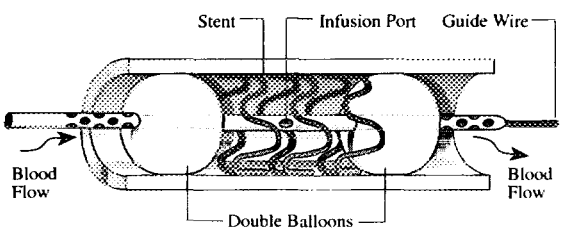


Figure 1. Diagram showing relationship of double-balloon perfusion catheter (USCI-Bard) to previously deployed stent. For simplicity, stretched areas of the vascular wall and recent neointimal growth are omitted.

analysis. All animal use was approved in advance by the Ochsner Institutional Animal Care and Use Committee.

Luciferase assays: Arterial segments and other organs (non-injured coronary artery, heart, kidney, liver, lung and spleen) were harvested at periods of 48 hours to three weeks following transfection with the luciferase constructs. Following administration of ~300 U/kg heparin to prevent thrombus formation and 100 mg/kg pentobarbital for euthanasia, the stented vessel was quickly placed in 4 °C phosphate buffered saline. The adventitia and stent was removed, and the tissue was minced and manually homogenized in an extraction buffer containing 1% Triton-X. Assays were performed according to Brasier et al. (17). Luciferin and purified luciferase were obtained from Analytical Luminescence Laboratory (San Diego, CA). Luciferase activity in relative light units (RLU's) was measured on an Analytical Luminescence Laboratory Monolight 2010 luminometer for a ten second integrated interval. Background was measured by mixing all components except tissue.

***In vivo* ganciclovir administration:** During the second surgery before gene transfer, a vascular access port (VAP; model GPV-5H-24, Access technologies) was implanted in swine receiving either the control or experimental construct as reported by Bailie et al. (18), with the following modifications. The catheter was trimmed to approximately 18-20 inches in length, and the catheter tip was advanced to the juncture of the inferior vena cava and right atrium using fluoroscopy for verification. If the tip was not clearly visible, then the catheter was flushed with a dilute contrast solution for visualization. Two days following gene transfer, Cytovene (Ganciclovir sodium; Syntex; Palo Alto, CA) administration began at a dose of 5 mg/kg iv (infused over one hour), every 12 hours for a period of two weeks using the following modifications of the technique reported by Bailie et al. (18). Following aseptic preparation of the skin, the sutured area was desensitized with a 10% lidocaine spray (Astra; Westborough, MA). A 20 gauge, 1.5" Huber point needle, attached to a one ml tuberculin syringe was passed into the septum of the access dome, and the heparin solution (50% dextrose + 500 U/ml heparin) remaining within the catheter was removed. The VAP was then flushed with 10 ml of sterile saline, and the Huber point needle connected to a 50 ml dextrose solution containing the required dose of ganciclovir. Following the infusion, another 10 ml of sterile saline was injected followed by 0.8 - 1.0 ml of dextrose-heparin solution.

Tissue harvest and processing: Four weeks following coronary artery injury, the animal was sedated and anesthetized as described above. The chest was opened along the sternum, and the major vessels (inferior and superior vena cava, pulmonary artery and aorta) were isolated and surrounded with size 0 silk suture. Following administration of heparin for anticoagulation (~400 Units/kg) and sodium pentobarbital for euthanasia (~100 mg/kg, iv), the isolated vessels were ligated (inferior vena cava and superior vena cava followed by pulmonary artery and aorta), and the heart was removed and transferred to room temperature saline. The appropriate coronary orifice was located, and the heart perfusion fixed with approximately 0.5 liter of 2% paraformaldehyde in 0.1M phosphate buffer (~37° C) at ~100 mmHg pressure for 30-45 min. Using fluoroscopy, the exact location of the stented arterial segment was located, which was then dissected free of the heart and trimmed of surrounding cardiac muscle and fat. The segments were stored in the same fixative at 4° C until processing. After the vessels were dehydrated and cleared, they were then infiltrated with Osteobed^R (Polysciences, Inc.). A Leitz 1600 Annular Sol Microtome with a tungsten-diamond blade (<300 μm, B-profile) was used to obtain serial sections (<20 μm) throughout the segment. Verhoeff's elastin stain was used for convenient visualization of the external and internal elastic laminae, followed by a picro/Ponceau counter stain every third or fourth section. Hematoxylin and eosin was occasionally used in adjacent sections for visualization of intimal, medial and adventitial cells when the internal and/or external elastic lamina was disrupted. The Optimas quantitative morphometry system was utilized to quantitate neointima formation (difference between the area bordered by the internal elastic lamina and the lumen area) and neointima/media ratio. The technician performing image analysis was blinded to the vessel category (experimental vs. control). A minimum of seven sections throughout the stented vessel segment were averaged to provide a single measure of neointimal area and neointima/media ratio for each stented vessel. Neointimal area and neointima/media ratio from control and experimental arteries were compared using unpaired t-tests. In addition, the sections were graded for injury score, percent stenosis, and neointimal thickness over the struts as described by Schwartz et al. (19). A p value <0.05 was considered significant.

RESULTS

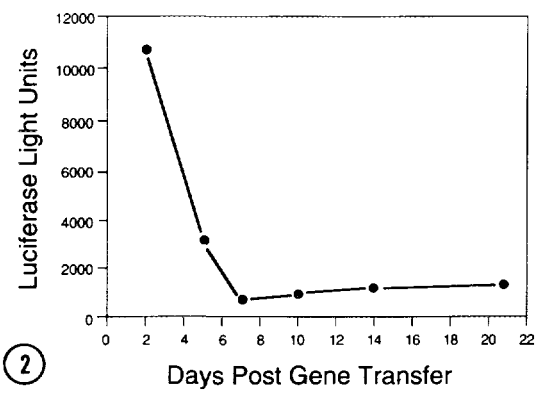
A total of 19 animals were utilized. Six were used to examine the strength and duration of marker gene (luciferase) expression, while 13 pigs received the retroviral HSVtk or control vector. One animal suffered bradyarrythmias following gene transfer and died approximately twelve hours later. Sections from another animal exhibited signs of reorganized thrombosis and were eliminated from the study.

Luciferase expression is exhibited in Figure 2. Maximal luciferase expression was observed two days following gene transfer, with a rapid decline to about 10% of these values by one week following gene transfer. However, even the lowest luciferase light units were more than two-fold over background values (403 $\pm$ 8 light units), and gene expression remained above background for at least three weeks. Gene expression was confined to the injured artery in all but one animal, in which several tissues (non-injured coronary artery, heart, kidney, liver and spleen) were positive (more than three standard deviations from background values) for luciferase ten days following gene transfer.

The in vitro effectiveness of ganciclovir against the HSV-tk producer cells is illustrated in Figure 3. Both producer lines exhibited exquisite sensitivity to ganciclovir. The LD_{50} (dose required to kill one-half the cells as assessed by cell counts) was 0.02 μM and 0.06 μM for LNS-TK and LNC-TK producing cells, respectively.

Quantitative analysis of the response to vascular injury following suicide or control vector insertion and ganciclovir treatment are illustrated in Figure four. There was no difference in neointimal or medial areas or neointima/media ratios between experimental and control groups. This lack of difference allowed us to combine groups for a further analysis of model characteristics.

Typical cross-sections showing neointimal proliferation following mild, moderate and severe injury are shown in Figure 5, while quantitative characteristics of the vessel injury response are depicted in Table 1 and Figure 6.



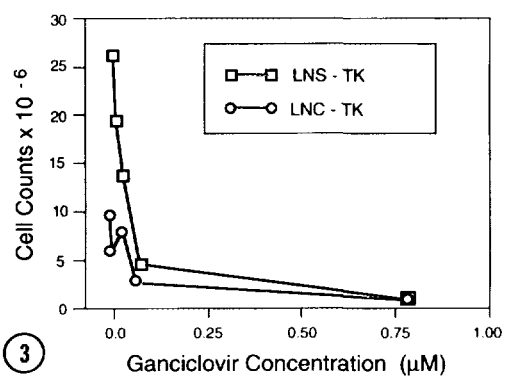


Figure 2. Marker gene (luciferase) expression as a function of time following gene transfer.

Figure 3. In vitro effectiveness of ganciclovir against two HSV-tk producing cell lines. Open squares represent the LNS-TK cells, while open circles represent LNC-TK cells.

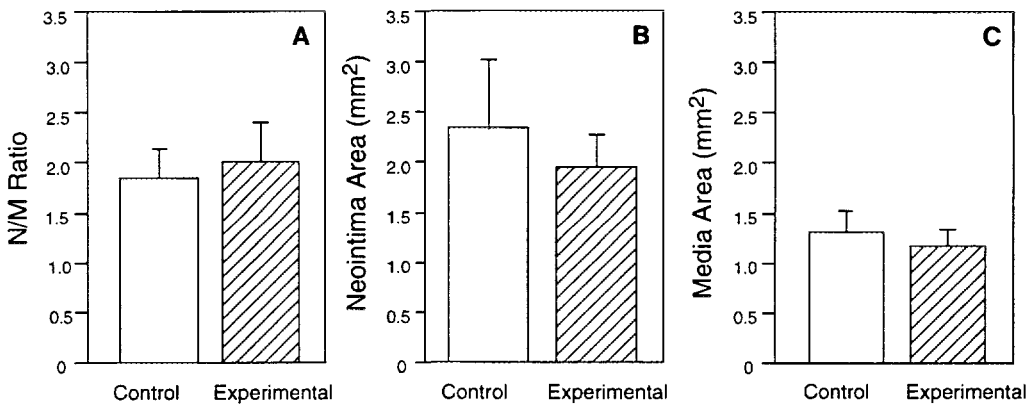


Figure 4. Comparisons of neointimal/media ratio, neointimal and medial areas between control and experimental groups. No significant differences were noted.

DISCUSSION

The primary vector systems utilized for direct *in vivo* gene transfer into the vascular wall include recombinant, replication-defective retroviruses or adenoviruses, and cationic liposomes. Retroviral vectors were one of the first systems used for gene transfer, demonstrating both long-term expression of gene products (14) and safety in human clinical trials (20). In addition, we found that retroviral-mediated delivery of the luciferase construct resulted in expression that was considerably higher than that seen with cationic liposomes following modified crush injury of canine peripheral arteries (21). Nevertheless, it is likely that only a few percent of vascular smooth muscle cells were infected. Several factors encouraged us to pursue a suicide gene approach with this system, despite the modest level of marker gene expression. First, retroviral vectors primarily target proliferating cells (22, 23). It is likely that the one week delay between

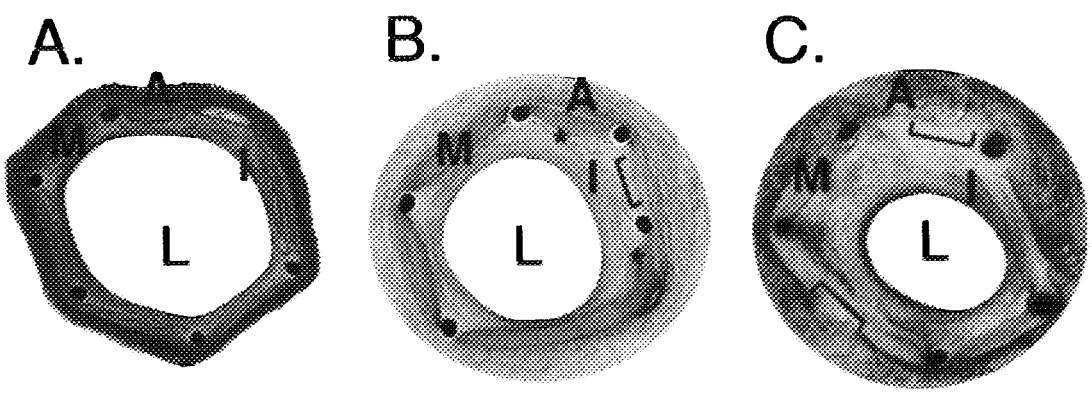


Figure 5. Typical sections of swine coronary arteries four weeks following stent deployment, showing mild (A), moderate (B) or severe (C) injury. Minimal disruption of the internal elastic lamina is seen in panel A. Medial thinning can be noted in the area enclosed by the bracket in panel B, while medial tearing is noted by the asterisks in panel B. Tears through the media and the external elastic lamina are shown enclosed by brackets in panel C. L - lumen; I - intima; M - media; A - adventitia.

Table 1. Comparison of response to vascular injury in present model vs. Schwartz et al. (19)

Group	Neointima (mm^2)	Media (mm^2)	N/M Ratio	% Stenosis	Neoint. Thick(mm)	Injury Score
Present findings	2.17 ± 0.38	1.26 ± 0.12	1.89 ± 0.23	49.5 ± 6.1	0.59 ± 0.08	1.6 ± 0.1
Schwartz et al.	2.42 ± 1.61	NA	NA	63	0.72 ± 0.33	1.9 ± 0.8

NA - data not available.

initial injury and gene transfer facilitated targeting of proliferating cells, since maximal VSMC division has been demonstrated at this time point in similar models (15, 16). Therefore, although a minority of medial and intimal VSMC were targeted, most of these were probably proliferating VSMC. Such delayed targeting may increase gene transfer efficiency compared to the low efficiency transfer seen in intact vessels with retroviral vectors (24). Second, inflammatory responses have been reported in target tissues following infection with recombinant adenovirus (25-27). We were concerned that such inflammatory processes might exacerbate VSMC proliferation, offsetting any gain from VSMC death following ganciclovir treatment. Finally, several studies have indicated that not only HSVtk transduced cells are killed after treatment with ganciclovir, but also surrounding cells (28). While the mechanism for this effect is unclear (29), such data led us to postulate that infection of a minority of proliferating VSMC might be sufficient to significantly attenuate neointimal formation following vascular injury. Nevertheless, our results indicate that the suicide vector strategy was not effective with our choices of vector and experimental animal model.

Since there were no significant differences between control and experimental animals, we combined the data from both groups and compared the histological characteristics of injury with other groups using oversized stents as a model of vascular injury. As shown in Table 1, our model is very similar to that reported by Schwartz et al. (19), with slightly smaller values for neointimal area, neointimal thickness over stent struts, percent stenosis and mean injury score.

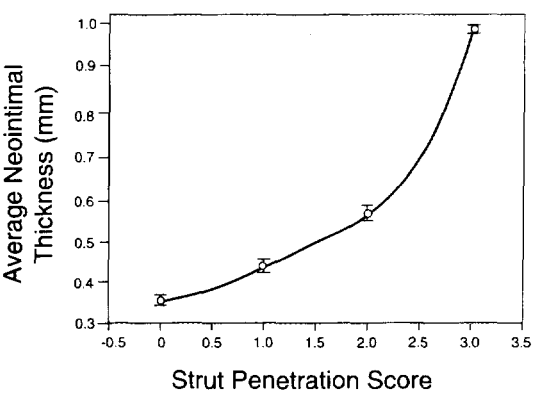


Figure 6. Relationship of neointimal thickness in mm over a given strut to the lumen versus injury score for that strut.

These minor differences may be due to the smaller inflation pressures utilized in this study (6 - 8 ATM) compared to those used by Schwartz et al. (8 - 10 ATM). Finally, we also found a curvilinear relationship between injury score for all sections analyzed and neointimal thickness over the stent struts, confirming that neointimal thickness and hence overall neointimal area increases rapidly with the rupture of the internal elastic lamina, and even more vigorously with tearing of the media (Figure 5, panels A-C, and Figure 6).

While this manuscript was in preparation, Nabel et al. (16) reported the successful use of a suicide vector approach to inhibit the response to vascular injury. These investigators utilized an adenoviral vector to deliver a HSVtk construct (expression regulated by the cytomegalovirus enhancer) immediately after balloon injury in peripheral arteries of juvenile swine. Other differences include the use of a five-fold higher dose of ganciclovir, albeit for a shorter period of time (six days in the Nabel et al. study vs. two weeks here). Neither the strength nor duration of HSVtk gene expression was reported. However, expression of the marker gene was demonstrated five days following gene transfer. No quantitative estimates of marker gene expression were provided. Assuming that Figure 2C (from the Nabel publication) is representative of the average marker gene expression, we estimate that ~10 % of the neointima and ~3 % of the media were stained positive for the marker gene (based on color threshold analysis of the scanned image). Thus, it is possible that the adenoviral vector utilized by Nabel et al. infected more medial VSMC than the retroviral vector used in this study.

In the Nabel model, the inhibition of neointimal growth with the use of the suicide vector vs. degree of vascular injury (one and five minute balloon inflation) deserves comment. The *in vivo* killing effect of ganciclovir following suicide vector deployment was considerably less following balloon inflation for five minutes vs. one minute. This change in balloon distension time resulted in a mere 15% increase in intima/media ratio. Nevertheless, the in vivo effect of ganciclovir was reduced from a 89% to 60% inhibition of intima/media ratio. The reason for this decreased effect with greater injury is unclear, but may be related to a VSMC density with increasing neointimal area (30), decreasing the "bystander effect" described earlier (28). Both of these models represent a mild degree of vascular injury, as opposed to our moderate to severe injury (intima/media ratio of 1.89). It is possible that moderate to severe neointimal proliferation would not be significantly affected by ganciclovir treatment following suicide vector deployment in this system. However, a decreased bystander effect could be offset by targeting a larger number of cells with an adenoviral vector. An adenoviral vector would likely target a larger number of VSMC in coronary arteries (31), due to the presence of a discontinuous rather than continuous internal elastic lamina, a physical barrier to viral particles in peripheral elastic arteries (32).

Some final comments regarding delivery systems for suicide vectors seems appropriate. Nabel et al. report impressive attenuation of neointimal growth without evidence of necrotic or inflammatory changes, and indicate that they have specifically targeted dividing VSMC. In a previous publication this group demonstrated that introduction of the HSVtk genome into normal rabbit arteries had no effect (11). In that study, a retroviral vector was utilized, which targets proliferating cells much more efficiently than quiescent cells (22, 23). Adenoviral vectors have

been shown in numerous studies to be either less specific toward the growth state of the target cell, or even more specific toward non dividing cells (33). Therefore, it is likely that a variety of cell types were targeted with the HSVtk construct. Nevertheless, the killing effect was probably specific to dividing cells, since incorporation of phosphorylated ganciclovir requires DNA replication.

In summary, the use of a retroviral system to deliver a suicide vector was not effective in decreasing the response to vascular injury in a swine model of coronary restenosis. However, this is a valid model to investigate the role of gene transfer in attenuating restenosis following injury. High efficiency adenoviral vectors may currently be the best tool for production of clinically relevant growth inhibitors or smooth muscle toxins to inhibit restenosis following angioplasty.

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