Regenerative Engineering and Translational Medicine (2023) 9:135–140
https://doi.org/10.1007/s40883-022-00272-z

ORIGINAL RESEARCH

Tissue-Engineered Endothelial Cells Induce Sustained Vascular Healing Through Early Induction of Vascular Repair

Sahil A. Parikh[1] · **Elazer R. Edelman**[2]

Received: 29 January 2019 / Revised: 28 July 2022 / Accepted: 30 July 2022 / Published online: 24 August 2022
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Abstract

Background Perivascular implantation of tissue-engineered endothelial cells (TEEC) after vascular injury profoundly inhibits neointimal hyperplasia. However, the time course and mechanism by which this effect occurs remain unknown. By developing genetically modified TEEC that express a "suicide gene," we can control the time during which the TEEC could exert their effect and determine the length of time TEEC need to be present following vascular injury to exert their inhibitory effect on long-term neointimal hyperplasia.

Methods Bovine aortic endothelial cells (BAE) were transfected with the human herpes simplex virus thymidine kinase (tk) gene to render them sensitive to ganciclovir (GCV). These BAE+tk were grown to confluence on Gelfoam and shown to have the same growth kinetics and biologic potency as control cells but were sensitive to GCV at low concentrations. The BAE+tk were grown on Gelfoam and placed in the perivascular space around balloon-injured rat carotid arteries. Rats randomly received BAE-tk, BAE+tk, or nothing (control) after balloon injury. GCV was administered early (days 1–7), late (days 5–11), or not at all.

Results Two weeks after injury, extensive neointimal hyperplasia was observed in control animals with an intima:media (I:M) area ratio of 0.80 ± 0.19. Early administration of GCV killed the BAE in constructs with TK sensitivity and eliminated the impact of TEEC regulation of intimal hyperplasia (0.45 ± 0.06). Intimal hyperplasia was still effectively reduced in animals with implants containing BAE-tk or BAE+tk which received GCV late (0.11 ± 0.04 and 0.19 ± 0.05). Immunohistochemistry demonstrated the lethal effect of GCV on TK-sensitive cells.

Conclusions The application of perivascular TEEC for only the first few days after injury had a significant inhibitory effect on intimal hyperplasia. This is in contrast to the results obtained in this same animal model with the infusion of isolated anti-smooth muscle cell proliferative agents. This suggests that the mechanism of action of TEEC may be upstream from smooth muscle cell proliferation. Moreover, the use of this technique to further elucidate biologic mechanisms will prove invaluable in the tissue engineering field.

Lay Summary We report a novel, genetically altered tissue-engineered endothelial cell (TEEC) implant that inhibits neointimal hyperplasia after experimental vascular injury. The viability of these implants can be carefully controlled and suggest a putative mechanism by which TEEC recapitulate control over the vascular response to injury.

Keywords Neointimal hyperplasia · Tissue engineering · Restenosis · HSV TK · Vascular injury

Introduction

Tissue engineering allows for the incorporation of large numbers of endothelial cells (EC) onto polymer scaffoldings [1, 2]. Tissue-engineered endothelial cells (TEEC) restore vascular health after injury and enhance repair [3–8]. Cells remain intact with normal growth kinetics, retention of immune identity, and biochemical secretory function [3]. When such devices are placed in cell culture, they perform like cultured cells, inhibiting binding of FGF2 to, and

✉ Elazer R. Edelman
ere@mit.edu

[1] Columbia University Vagelos College of Physicians and Surgeons, 161 Fort Washington Avenue, 6th Floor, New York, NY 10032, USA

[2] Harvard-MIT Division of Health Sciences and Technology, MIT E25-438, 77 Massachusetts Avenue, Cambridge, MA 02139, USA

proliferation of, vascular smooth muscle cells (SMC) [3]. When these same devices are implanted around injured blood vessels, they offer the greatest control over vascular injury through paracrine mechanisms independent of vEGF [3]. The implantation of tissue-engineered porcine aortic endothelial cells (PAEC) within Gelfoam matrices around injured porcine carotids resulted in nearly a 50% reduction in intimal hyperplasia [7]. The vascular response to injury occurs in a stereotypic stepwise progression (thrombosis then inflammatory cell recruitment then smooth muscle cell proliferation then vascular remodeling) [9]. The question arises as to whether TEEC need be present perpetually or for only a brief window of vulnerability after vascular injury and until healing. We hypothesize that there may be a window of opportunity immediately after vascular injury when TEEC exert a reparative influence which will remain long after these cells are no longer present. Understanding the critical time window when TEEC exert their influence may implicate a putative mechanism of action for the implants and direct further study.

To approach this question, we constructed an experimental system in which the expression of TEEC gene products was regulated temporally through time-programmed cell ablation. Coordinated cell death was achieved by transfecting the ECs with a "suicide" gene, the Herpes Simplex Virus thymidine kinase (HSV tk) as has been previously described [10]. EC with the HSV tk phosphorylate the nucleoside analog ganciclovir to a monophosphate, which is itself converted to a nucleoside triphosphate via native kinases and, once incorporated into DNA via native replication machinery, terminates DNA chain propagation, inducing cell death via apoptosis [10]. Systemic administration of ganciclovir should reproducibly ablate TEEC implants at precise times after implantation [11–13].

We examined the stability of EC transfected with the HSV tk gene, their secretion of bioregulatory compounds, their ability to modulate controlled vascular injury, and their response to ganciclovir programmed suicide. The results not only extend the armamentarium of tissue engineering as a research tool and therapeutic modality but add further insight to the nature of vascular repair.

Materials and Methods

In Vitro: Cell Isolation, Transfection, Characterization, and Engraftment

Bovine aortic endothelial cells (BAEC) were isolated and cultured at passage 8 or lower [3]. Some BAEC were transfected via lipofection with Lipofectamine (Gibco/Invitrogen, Carlsbad, CA) with a commercially available plasmid vector that carried the HSV tk gene under the control of a CMV promoter with a resistance gene for selection (HSV+TK) and others with the same plasmid vector without the HSV tk gene (HSV-TK) (InvivoGen, San Diego, CA) [6].

Following transfection and selection, the cells were passaged in Dulbecco's modified Eagle's medium (DMEM — Gibco/Invitrogen) supplemented with 10% fetal bovine serum (FBS — Gibco/Invitrogen) and the appropriate selection agent (Zeocin/Zeocell — InvivoGen). Cell morphology was assessed daily with light microscopy. Growth kinetics, protein content, and production of heparan sulfate proteoglycans were assessed as previously described [6]. BAEC ± HSV TK susceptibility to ganciclovir was verified in vitro by graded exposure to ganciclovir for 48 h using a standard aqueous cell viability assay (Promega, Madison, WI).

BAE±TK (1×10^{-5} cells) were suspended in media (100 µl of DMEM with penicillin (100 U/mL), streptomycin (100 µg/mL), glutamine (2.0 mmol/L), and 10% fetal calf serum (Life Technologies, Inc.) with three-dimensional scaffolds of denatured collagen ($1.5 \times 1.0 \times 0.3$ cm^3 sterile Gelfoam, Pfizer, NY, NY). Cells were allowed to attach for 2 h before the cell loaded Gelfoam blocks were placed in 17 × 100-mm polypropylene tubes containing 2 mL of culture media. Cells were cultured for 14 days in 10% CO_2/90% O_2, and the medium was changed every 72 h. The number of cells attached to the Gelfoam was determined after digestion with collagenase (Worthington Biochemical Corp., Freehold, NJ), and cell viability was assessed by trypan blue exclusion. Endothelial cells were cultured to confluence within the blocks before implantation. Total protein, glycosaminoglycan (GAG), and heparan sulfate proteoglycan (HSPG) production was assayed as previously described [4, 5].

In Vivo: Balloon Injury, TEEC Implantation, and Histopathology

Studies were completed in AAALAC-accredited facilities staffed by certified veterinarians and supervised by the Division of Comparative Medicine in accordance with the standards in the Guide for the Care and Use of Laboratory Animals and University oversight protocols. Endothelial denudation of the left common carotid artery of Sprague Dawley rats was performed with passage of a 2 French Fogarty balloon catheter (Baxter, Deerfield, IL) via an external carotid arteriotomy into the proximal common carotid artery [3, 6]. The balloon was inflated and withdrawn thrice along the length of the artery. On removal of the catheter, the external carotid was ligated just proximal to the arteriotomy. Gelfoam blocks containing BAE-TK, BAE+TK, or no cells were then wrapped around the injured artery. Animals were administered ganciclovir (5 mg/

kg intraperitoneally) or an equal volume of normal saline twice daily for prespecified intervals: group 1 — BAEC+TK (ganciclovir days 1–7 — $n = 11$), group 2 — BAEC-TK (ganciclovir D1–7 — $n = 8$), group 3 — BAEC+TK (ganciclovir D5-11 — $n = 4$), group 4 — BAEC+TK (no ganciclovir — $n = 4$), group 5 — balloon injury alone (no ganciclovir — $n = 8$). In a second experimental group, balloon injury was performed on day 0, and the EC/Gelfoam wraps were implanted during a second surgery on day 5.

On the 14th post operative day, all the animals were euthanized, and the carotid arteries were harvested with the Gelfoam implants intact. Arteries were fixed in 10% neutral buffered formalin and divided into four equal segments, which were paraffin embedded and microtome sectioned. Four to six 8-µm sections along the length of each segment were obtained and stained with hematoxylin-eosin or Verhoeff's elastin stain. The intimal and medial areas were determined for each arterial segment by using computerized digital planimetry with a dedicated video microscope and customized software (Adobe Photoshop and NIH Image). Intimal hyperplasia was quantified by the I:M ratio. Artery/Gelfoam sections were also stained with antibodies against Factor VIII (Dako, Denmark) to visualize the EC remaining within the Gelfoam.

Statistics

All data are presented as means ± SEM. Statistical analysis comparing treatment groups used a non-paired Student's t test with the Bonferroni correction. Values of $p < 0.05$ were considered statistically significant (Microsoft Excel, Microsoft, Redmond, WA).

Results

In Vitro

Following lipofection, BAE+TK produced similar amounts of protein, GAG, and HSPG to control BAE (Fig. 1A and B). BAE±TK were then grown to confluence on Gelfoam blocks and demonstrated similar growth kinetics and cell viability to control BAE (Fig. 1C). BAE+TK demonstrated significant cytotoxicity to ganciclovir when exposed to doses greater than 5 mcg/mL whereas BAE-TK did not have any significant retardation in growth kinetics at doses of 10mg/mL (data not shown). These results were recapitulated in BAE+TK and BAE-TK when grown on Gelfoam blocks and exposed to identical doses of ganciclovir in culture (Fig. 1D).

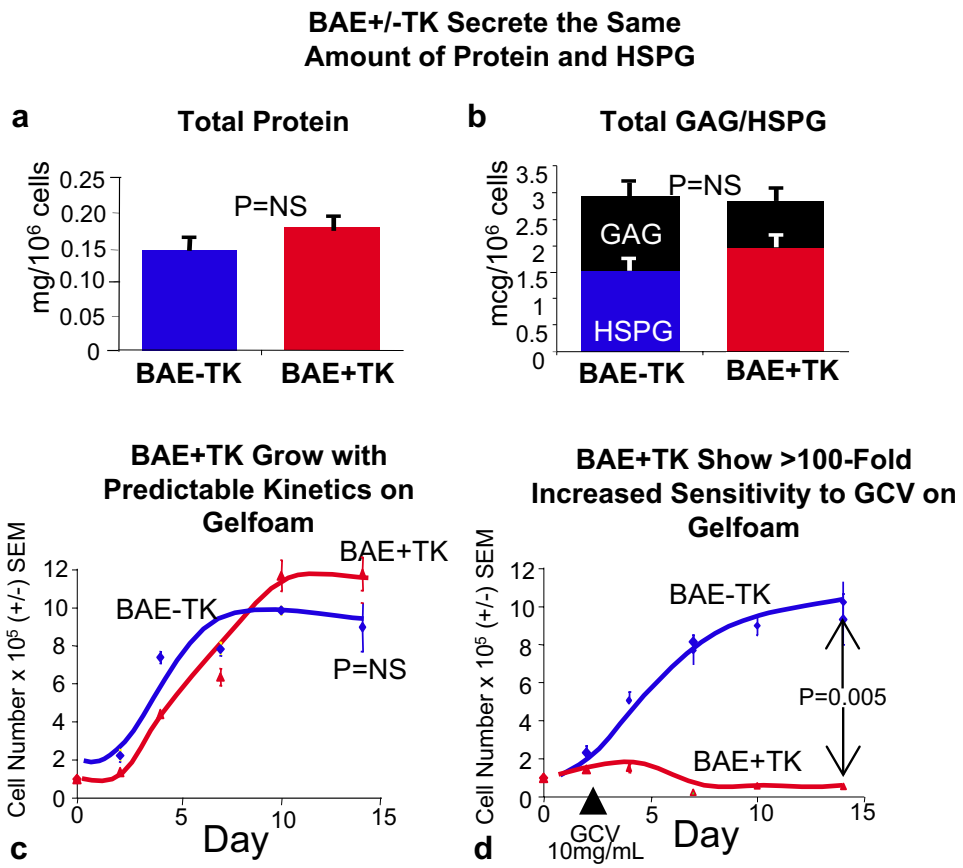


Fig. 1 Bovine aortic endothelial cells (BAE) lipofected with control plasmid (−TK) or HSV tk (+TK) expressed the same amount of total protein (A) and glycosaminoglycans (GAG) or heparan sulfate proteoglycans (HSPG) (B) after up to 8 passages. These same cells were noted to grow with predictable, normal growth kinetics in typical cell culture medium (C). However, in the presence of ganciclovir (10 mg/mL), BAE-TK grew with normal kinetics whereas BAE+TK cultures underwent apoptosis and rapidly lost viability

In Vivo

Experimental groups spanned the complete course of TEEC exposure and possible combinations of ganciclovir administration and TEEC implantation. Placement of TEEC constructs with and without TK sensitivity at the time of endothelial-denuding injury significantly reduced the resultant intimal hyperplastic reaction; intima:media (I:M) ratios fell from 0.82 ± 0.12 to 0.11 ± 0.04 and 0.16 + 0.–5 (Fig. 2, $p < 0.01$). However, if the TEEC implants were placed 5 days or more after injury, or if ganciclovir was administered to BAE+TK early after implantation, there was no beneficial effect (I:M 0.73 ± 0.04 and 0.45 ± 0.06, NS compared with control), suggesting that intact EC were needed early after injury, during a specific period of vulnerability and healing and not thereafter. Indeed, administration of ganciclovir to animals with BAE+TK implants after this time window had no effect on reduction of hyperplasia as I:M ratios remained suppressed (0.19 ± 0.06).

Immunohistochemistry demonstrated the lethal effect of ganciclovir on BAE+TK cells in a qualitative manner. In implants removed at the time of animal sacrifice, immunostaining against Factor VIII, specific to EC, demonstrated significantly more remaining endothelial cells on BAE-TK implants compared to BAE+TK implants after both had been exposed to ganciclovir (Fig. 3).

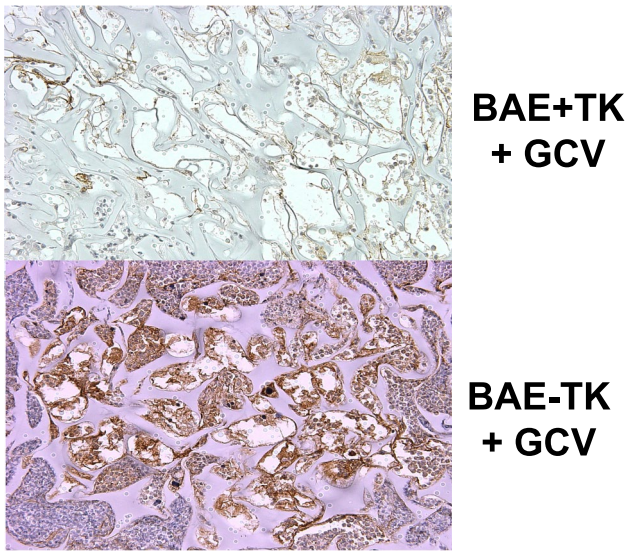


Fig. 3 TEEC implants were removed intact 14 days after implantation and were fixed and embedded. Staining with anti-factor VIII antibody (brown) demonstrates the dramatic reduction in remnant BAE+TK after ganciclovir exposure compared to BAE-TK

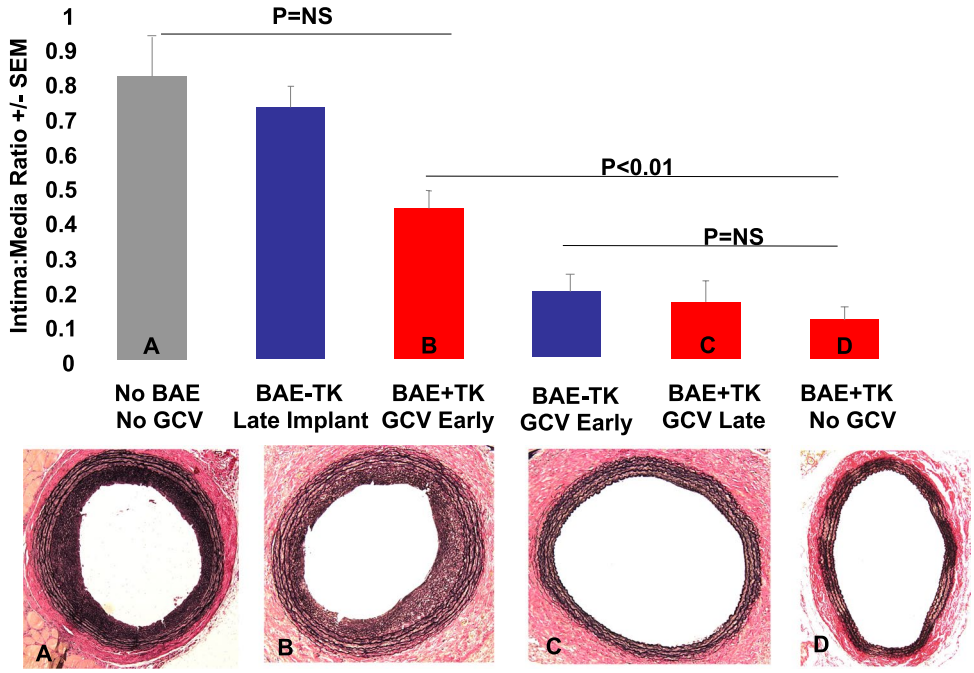


Fig. 2 Experimental groups spanned the complete course of TEEC exposure and possible combinations of ganciclovir administration and TEEC implantation. The intima:media ratio (I:M) is depicted for each treatment. In animals with balloon injury alone, a significant neointima with I:M ratio of 0.82 ± 0.12 was noted 14 days after injury (**A**). Placement of TEEC with and without TK sensitivity at the time of endothelial-denuding injury significantly reduced the resultant intimal hyperplastic reaction; intima:media (I:M) ratios fell to 0.11 ± 0.04 (**D**) and 0.16 ± 0.05 (**C**) ($p < 0.01$). If TEEC implants were placed 5 days or more after injury, or if ganciclovir was administered to BAE+TK early after implantation (**B**), there was no beneficial effect (I:M 0.73 ± 0.04 and 0.45 ± 0.06, p = NS compared with control), suggesting that only the presence of TEEC early after injury reduced intimal hyperplasia. Administration of ganciclovir to animals with BAE+TK implants after this time window had no effect on reduction of hyperplasia as I:M ratios remained suppressed (0.19 ± 0.06)

Discussion

Our results demonstrate that it is technically feasible to stably transfect BAE with the HSV tk gene with a lipofection technique and select these cells in vitro with stable gene expression through 8 passages. The transfection of BAE with HSV tk in this model did not significantly alter the biochemical profile and activity of the cells as demonstrated by the finding that both BAE+TK and BAE-TK expressed similar amounts of protein, GAG, and HSPG as control cells. In addition, growth kinetics and cell viability remained unaltered. More importantly, our in vitro studies revealed that BAE+TK were exquisitely sensitive to low concentrations of ganciclovir. This result was critical to the feasibility of our in vivo studies, as relatively low systemic doses of ganciclovir were sufficient to achieve tissue concentrations of drug toxic to BAE+TK implants.

The in vivo experiments demonstrate that BAE±TK inhibit intimal hyperplasia to a significant extent, comparable to prior studies in our lab with control, wild-type BAEC. However, the early administration of ganciclovir from days 0 to 5 to BAE+TK-treated subjects abrogated the inhibitory effect of these TEEC implants on neointimal hyperplasia. The administration of ganciclovir late — days 5 to 10 — after injury had no significant impact on TEEC inhibition of neointimal hyperplasia. These findings are corroborated by the late implantation of TEEC on day 5 in the supplementary experiment. These data strongly support the existence of a window of vascular vulnerability early after injury and the permanence of healing once this window is survived. This early period sees the most dramatic inflammatory cell recruitment and early SMC proliferation and migration in the rat carotid model [14–19]. Cell-based constructs are therefore an ideal therapy for such delimited injury. In stark contradistinction to pharmacologic agents which inhibit vascular reactivity without restoring health [20], cell-based therapies provide immediate intervention without added injury, promote healing and inducing repair rather than staying inevitable processes, and can then be made to disappear without adverse reactivity. The use of three-dimensional constructs for cell seeding adds reduced immunogenicity and inflammation [21, 22]. Free cell implantation induces a brisk immune response that can even be seen with autologous cells given the high levels of circulating anti-EC antibodies seen in vascular diseases [23]. The matrix-embedded TEEC shield antigenic surface of EC and can offer immune shielding for even xenogeneic implants [5, 8, 21–25]. It is plausible that TEEC influence vascular repair by mitigating inflammatory cell recruitment to the vascular lumen which in turn halts the stimulus for SMC proliferation and migration.

Finally, the application of suicide gene therapy in tissue engineering may have broad implications for the utilization of TEEC and other biosynthetic devices. The tailored expression of a cellular component's paracrine effectors and secretory products may well enable cell types to be combined to recapitulate complex biochemical environments for precisely controlled periods of time may be possible. The combination of tissue engineering and vascular biology offers new research tools and therapeutic options.

Acknowledgements The authors acknowledge the technical contributions of Helen M. Nugent, PhD, Amy C. Lee, MD, Adam Groothuis, PhD, Brad C. Carofino, MD, and Philip Seifert, BS, in the performance of these experiments.

Funding Open Access funding provided by the MIT Libraries Dr. Parikh was supported by the Sarnoff Endowment for Cardiovascular Science for initial work and later by NIH T32 HL007604. This work was supported by NIH R01 HL060407 awarded to Dr. Edelman.

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