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5. The endothelial cells exhibited growth characteristics and morphology typical of porcine endothelium in tissue culture. They also expressed receptors for the acetylated form of low density lipoprotein (AcLDL), in contrast to fibroblasts and other mesenchymal cells [R. E. Pitos, T. L. Innerarity, J. N. Weinstein, R. W. Mahley, *Arteriosclerosis* **1**, 177 (1981); T. J. C. Van Berkel, J. F. Nagelkerke, J. K. Kruijt, *FEBS Lett.* **132**, 61 (1981); J. M. Wilson, D. E. Johnston, D. M. Jefferson, R. C. Mulligan, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 4421 (1988)]. More than 95% of the cultured cells contained this receptor, as judged by fluorescent AcLDL uptake. Cell cultures were incubated with (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbacyanine perchlorate) (Dil) AcLDL (Biomedical Technologies) (10 μg/ml) for 4 to 6 hours at 37°C, followed by three rinses with phosphate-buffered saline containing 1% paraformaldehyde and visualized by phase-contrast and fluorescent microscopy to detect AcLDL uptake. These cells also contained von Willebrand factor, detected in cDNA by the polymerase chain reaction.
6. Endothelial cells containing this vector were selected for their ability to grow in the presence of G418. Greater than 90% of selected cells synthesized β-galactosidase by histochemical staining (Fig. 2B). The endothelial cell nature of these genetically altered cells was also confirmed by analysis of fluorescent AcLDL uptake and von Willebrand factor detected in cDNA by using the polymerase chain reaction. Infection by BAG retrovirus was further verified by DNA blot analysis, which revealed the presence of intact proviral DNA at approximately one copy per genome.
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Implantation of Vascular Grafts Lined with Genetically Modified Endothelial Cells

JAMES M. WILSON, * LOUIS K. BIRINYI, ROBERT N. SALOMON, PETER LIBBY, ALLAN D. CALLOW, RICHARD C. MULLIGAN

The possibility of using the vascular endothelial cell as a target for gene replacement therapy was explored. Recombinant retroviruses were used to transduce the *lacZ* gene into endothelial cells harvested from mongrel dogs. Prosthetic vascular grafts seeded with the genetically modified cells were implanted as carotid interposition grafts into the dogs from which the original cells were harvested. Analysis of the graft 5 weeks after implantation revealed genetically modified endothelial cells lining the luminal surface of the graft. This technology could be used in the treatment of atherosclerosis disease and the design of new drug delivery systems.

MYOCARDIAL INFARCTIONS, strokes, and amputations due to lower extremity occlusive disease are the clinical sequelae of atherosclerosis and the major causes of mortality in our society despite concerted attempts at prevention and treatment (*1*). One important clinical intervention in advanced disease states is the replacement of stenotic or occluded arteries with bypass grafts. In large diameter vessels, this can be accomplished through the use of autologous veins or arteries or by using prosthetic conduits composed of materials such as dacron or expanded polytetrafluoroethylene (*2*). Bypass of smaller diameter, low flow vessels (for example, coronary arteries and some peripheral arteries) usually requires the use of autologous vessels, since the performance of prosthetic vascular grafts is poor (*2*).

We have considered whether the performance and versatility of prosthetic vascular grafts might be improved by lining the luminal surface with endothelial cells genetically modified to promote repopulation, prevent thrombosis, or secrete therapeutic proteins. As a first step, we implanted into dogs vascular grafts seeded with autologous retrovirus-transduced endothelial cells by means of a technique that has been used in the study of graft thrombosis (*3*). External jugular veins harvested from adult mongrel dogs were used as a source of endothelial cells. The cells were plated in culture and expanded in vitro for 10 to 14 days. Cells from each animal were divided into two portions and infected with a replication-defective retrovirus or were mock-infected. Small diameter Dacron grafts were seeded at subconfluent densities with endothelial cells and surgically implanted as carotid interposition grafts into the dog from which the cells were harvested. Each dog received a graft seeded with the genetically modified cells (subsequently referred to as the genetically modified graft) and a contralateral graft seeded with mock-infected cells. Five weeks after implantation the grafts were harvested and analyzed.

Replication-defective retroviruses with amphotropic host range were used to introduce a reporter gene into the genomic DNA of the endothelial cells. The *Escherichia coli lacZ* gene was used as the reporter gene because its product of expression, β-galactosidase, can be detected in situ through the use of enzymic histochemical assays that stain the cell's cytoplasm blue (*4*). Two retroviral vectors that express the *lacZ* gene, BAG and BAL, were used in this study (*5*). Viruses derived from these vectors were used to infect primary cultures of endothelial cells derived from each dog. Approximately 5% to 15% of the endothelial cells exposed to the BAG virus were infected, whereas 40% to 60% of the endothelial cells exposed to BAL virus were infected (Table 1).

At the time of seeding, the cultures were analyzed for endothelial cell–specific function [uptake of acetylated low density lipoprotein (LDL) and the presence of von Willebrand's factor] and for the presence of antigens specific for smooth muscle cells (α- and γ-actin isoforms). These analyses indicated that >98% of the cells from each isolate were functional endothelial cells.

Dogs 1 to 3 received implants seeded with BAG-infected endothelial cells, whereas dogs 4 to 7 received implants seeded with BAL-infected endothelial cells. After 5 weeks, the grafts were explanted and divided longitudinally into thirds for analysis. The analyses included fixation and scanning electron microscopy, fixation and histochemical characterization for β-galactosidase–ex-

J. M. Wilson and R. C. Mulligan, Whitehead Institute and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02142.
L. K. Birinyi, R. N. Salomon, P. Libby, A. D. Callow, Departments of Surgery and Pathology and Medicine, Tufts University New England Medical Center, Boston, MA 02111.

*Present address: Howard Hughes Medical Institute, Departments of Internal Medicine and Biological Chemistry, University of Michigan Medical School, Ann Arbor, MI 48109.

pressing cells, and enzymatic release of luminal cells from the graft material for cytochemical characterization in vitro. Six of seven dogs had bilaterally patent grafts at 5 weeks. Scanning electron microscopy showed a lining of cells with endothelial-like morphology on the luminal surface of 10 of 12 patent grafts; however, the endothelial cell monolayer was variably disrupted along the peaks of the crimped grafts during the harvesting process.

Each patent and repopulated graft that had been seeded with infected endothelial cells contained β-galactosidase–positive cells on the lumen of the vessel (Fig. 1). Contralateral grafts seeded with mock-infected endothelial cells showed no stained cells. Regional variation in the density of genetically modified cells was seen in each graft but was independent of proximity to distal or proximal anastomosis. Sections of the graft that were completely devoid of genetically modified cells usually corresponded to areas in which the monolayer had detached during explantation.

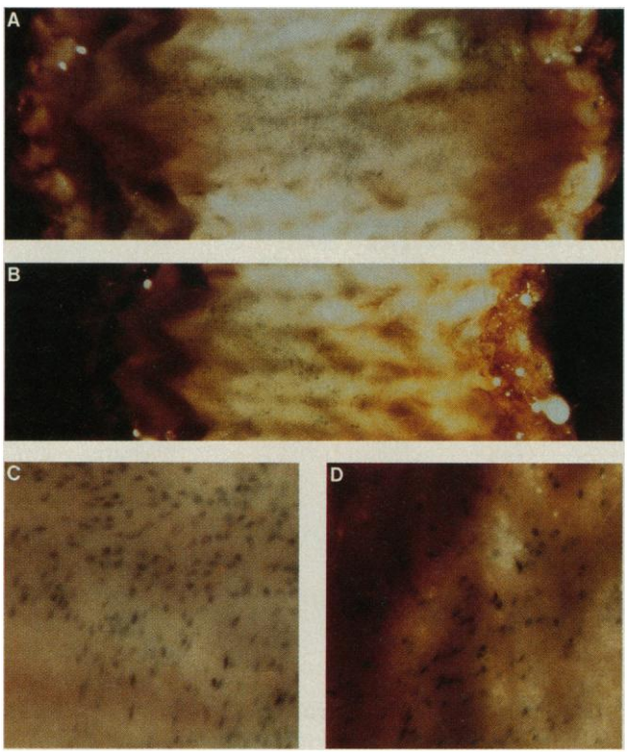

Fig. 1. In situ analysis of grafts for virus-transduced cells. A longitudinal section of the genetically engineered implant from animal 6 was analyzed for cells that express virus-directed β-galactosidase. Grafts were cut longitudinally into thirds and a third was fixed for 5 min in phosphate-buffered saline (PBS), *p*H 7.4, containing 0.5% gluteraldehyde, washed in PBS three times, and incubated in X-gal solution for 2 hours (*4*). The grafts were visualized en face through a Leitz dissecting microscope and two representative fields are presented in (**A**) and (**B**) at ×30 magnification. Flow of blood is from top to bottom. Selected areas in (A) and (B) are presented at higher magnifications (×100) in (**C**) and (**D**), respectively.

Cells were enzymatically harvested from the luminal surface of selected grafts to permit more detailed characterizations of cellular composition and function. Primary cultures of cells were established and expanded in vitro for approximately 2 weeks before being analyzed for endothelial cell–specific function and expression of viral-directed β-galactosidase. Most of the cells that were harvested from explants and expanded in vitro retained differentiated endothelial cell functions; however, smooth muscle cells eventually overgrew one of the cultures. Cells expressing viral-directed β-galactosidase were detected in all cultures established from genetically modified grafts. Genetically modified cells from explant-derived cultures were detected at 25% to 50% of the amount used for seeding. The lower number of genetically engineered cells released from the explant can be explained by a selective disadvantage conferred onto endothelial cells by retroviral transduction, by partial repopulation of the seeded graft with endogenous endothelial cells, by nonrepresentative expansion of the cells isolated from the graft surface, or by shutoff of expression of β-galactosidase in a portion of the cells. Although these explanations are not mutually exclusive, partial repopulation with endogenous endothelial cells is most likely in light of the composition of the grafts (*6*).

In this study, we demonstrated the feasibility of implanting vascular grafts seeded with autologous genetically modified endothelial cells. The cells are present on the lumen of the graft for at least 5 weeks and the transferred gene continues to function. This technology has potential applications for the treatment of atherosclerotic disease and the design of new drug delivery systems. Retrovirus-mediated transduction of endothelial cells could be used to improve the function of vascular grafts in ways not previously possible. Endothelial cells could be genetically modified to secrete proteins capable of preventing thrombosis or inhibiting neo intimal smooth muscle cell hyperplasia, two common pathological mechanisms of graft failure (*2*). Our results also suggest that endothelial cells may be an appropriate target cell for gene transfer when designing drug delivery systems. Because of their prox-

Table 1. Summary of implantation experiments. Autologous endothelial cells were harvested from external jugular veins of mongrel dogs (20 to 25 kg) by previously described methods (*3*), plated into fibronectin-coated flasks, and maintained in M199 medium supplemented with 5% plasma-derived equine serum, penicillin, streptomycin, porcine heparin (100 μg/ml), and endothelial cell growth factor during two serial passages over a 10- to 14-day period. During this time period, the cells were exposed to fresh stocks of virus supplemented with polybrene (8 μg/ml) every 3 days (18 hours per exposure). At the end of the second passage, cells were harvested and samples were analyzed directly, cryopreserved, or used to seed 6 cm by 4 mm knitted Dacron grafts (CR Bard, Billerica, MA) according to the four-step method of Yates in which 0.75×10^6 endothelial cells were added to the autologous blood during the second and third steps (*8*). Animals were anesthetized, and 6-cm segments of both carotid arteries were replaced with the seeded grafts as described (*3*). Each animal received an implant seeded with infected endothelial cells and a contralateral graft seeded with mock-infected cells. Five weeks later the animals were anesthetized and the grafts were removed and analyzed as described in the legend to Fig. 1. Efficiency is the efficiency of infection of endothelial cells used for seeding, as determined by the in situ cytochemical stain for β-galactosidase. Coverage represents the percentage of luminal surface area lined with endothelial cells as determined by scanning electron microscopy; this represents a conservative estimate of actual coverage since portions of the monolayers were detached during the fixation of the explant. NA, not analyzed.

Animal	Retroviral infection		Analysis of explants			
			Patency		Coverage	
	Virus	Efficiency	Infected	Mock	Infected	Mock
1	BAG	15%	Yes	Yes	>85	>85
2	BAG	5%	Yes	Yes	>85	>85
3	BAG	5%	Yes	Yes	>85	>85
4	BAL	40–60%	No	No	NA	NA*
5	BAL	40–60%	Yes	Yes	>15	>15
6	BAL	40–60%	Yes	Yes	50–60	>85
7	BAL	40–60%	Yes	Yes	>85	>85

*Not analyzed because the graft was thrombosed.

imity to the blood stream endothelial cells are an obvious candidate for delivering therapeutic proteins systemically. Genetically engineered vascular grafts could also be used to target the delivery of a therapeutic protein to a specific organ or limb that is perfused by blood passing through the grafted artery. Specific applications might include the secretion of vasodilators or angiogenic factors to ischemic myocardium or the delivery of an antineoplastic agent to an organ riddled with metastatic tumor.

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5. The BAG vector, which expresses β-galactosidase from a transcript initiated at the 5′ long terminal repeat (LTR), has been described (*4*). The BAL vector is derived from the previously described BA-LDLR vector [J. M. Wilson, D. E. Johnston, D. M. Jefferson, R. C. Mulligan, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 4421 (1988)], except that low density lipoprotein receptor (LDLR) cDNA sequences were replaced with coding sequences for the *lacZ* gene. Expression of *lacZ* from this vector is driven from a transcript that it initiated at β-actin promoter sequences located internal to the viral transcript. Each vector was transfected into the amphotropic packaging cell line ψ CRIP [O. Danos and R. C. Mulligan, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6460 (1988)], and individual transfectants were analyzed for production of recombinant virus. Viral titer was measured by exposing subconfluent plates of NIH 3T3 cells to limiting dilutions of viral stocks and subsequently analyzing the confluent fibroblasts for clones of β-galactosidase–expressing cells (*4*). Titers of the best virus-producing cell lines were 0.5×10^5 to 1×10^5 colony-forming units (CFU) per milliliter for BAG-derived virus and 2×10^5 to 5×10^5 CFU per milliliter for BAL-derived virus. Virus-producer cell lines and infected populations of endothelial cells were free of helper virus.
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Control of Gene Expression by Artificial Introns in *Saccharomyces cerevisiae*

TADANORI YOSHIMATSU AND FUMIKIYO NAGAWA*

Artificial yeast introns that show cold-sensitive splicing have been constructed. These conditional introns can be inserted into a target gene as an "intron cassette" without disrupting the coding information, allowing expression of the gene to be cold sensitive. Insertion of these intron cassettes rendered the yeast *URA3* gene cold sensitive in its expression. The advantage of this intron-mediated control system is that any gene can be converted to a controllable gene by simple insertion of an intron.

ONLY A SMALL NUMBER OF GENES in *Saccharomyces cerevisiae* contain introns, which are usually found individually near the 5′ end of their genes (*1*). The role of such rare introns is not clear. The removal of an intron from the yeast actin gene did not affect its expression (*2*). Nevertheless, they do appear to have a regulatory function in some cases. For example, splicing of an intron in the yeast ribosomal gene *RPL32* is autogenously controlled by its product (*3*).

Each yeast intron contains three conserved sequences, GUAPyGU (Py, pyrimidine) at the 5′ donor end, UACUAAC at the branch point, and PyAG at the 3′ acceptor end (*4*). Base pairing between small nuclear RNAs (snRNAs) and GUAPyGU and UACUAAC sequences is thought to be important for efficient splicing (*5*). These consensus sequences are similar to those found in higher eukaryotes. However, the sequence requirement is more stringent in yeast than in higher eukaryotes (*6*). In addition, there are no conserved sequences in the flanking exons of yeast.

Heterologous introns have been successfully inserted into target genes in order to study mRNA splicing (*2, 3, 5, 7, 8*). The same strategy was also used to study Ty transposition (*9*) and activation at upstream sites (*10*). However, in these investigations, an intron was inserted along with its flanking exon sequences. These flanking sequences can cause inactivation of the target gene. Therefore, we have inserted an artificial intron totally devoid of flanking exon sequences and have used it to control the heterologous gene expression.

The intron consisted of 30 bp of the 5′ end of the yeast *RP51A* intron (*7*), 30 bp of the 3′ end of the yeast *S10* intron (*11*), and 44 bp of polylinker sequence of an M13 cloning vector (Fig. 1). This intron contains all the necessary sequences for splicing in yeast. In addition, we have placed Sna BI and Pvu II restriction sites at the ends. Since the Sna BI and Pvu II recognition sequences overlap with the three terminal bases at each intron end, the artificial intron can be cleaved out precisely as a "cassette" without any additional exon sequences. This intron fragment can be inserted into any gene without disrupting its coding information by cutting the gene with a blunt end–producing restriction enzyme and then inserting the intron fragment. Alternatively, if the restriction enzyme leaves a protruding 5′ terminus, the fragment can be treated at one end with single strand–specific nuclease and at the other end by the Klenow fragment of DNA polymerase I.

To show that the artificial intron can be inserted into a gene without disrupting its function, we inserted it into the *URA3* coding region at Eco RV, Sca I, or Stu I sites (*12*), which are located 186, 310, and 434 bp downstream of the start codon, respectively. These intron-containing *URA3* genes were located on a "2μ plasmid" construct (*13*). This construct was able to complement a *ura3-52* mutation on the yeast chromosome (*14*), which suggests that the artificial intron is spliced out precisely and

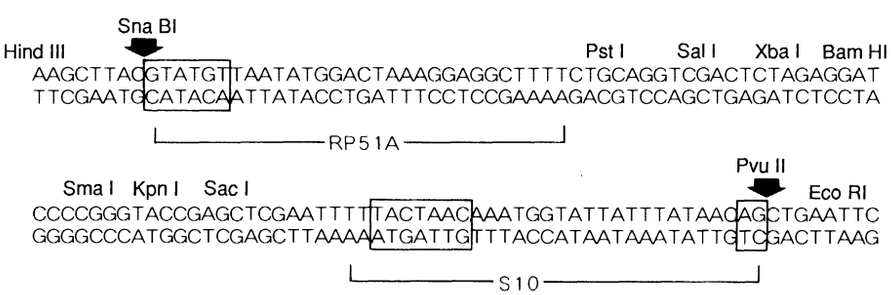

Fig. 1. Structure of the artificial intron. The 5′ end fragment and the 3′ end fragment were chemically synthesized, and the polylinker fragment (from Pst I to Sac I) was obtained from plasmid pUC18. The consensus sequences are shown in boxes. This intron was cloned in the Hind III–Eco RI site of pUC18 to generate pUC-AI. The 5′ end fragment of the yeast *RP51A* intron and the 3′ end fragment of the *S10* intron are indicated.

Molecular Biology Section, Institute for Biotechnology Research, Wakunaga Pharmaceutical Co., Ltd., 1624 Shimokotachi, Koda-cho, Takata-gun, Hiroshima, 729-64, Japan.

*To whom correspondence should be addressed.