

Analysis of VNTR loci amplified by the polymerase chain reaction for investigating the origin of intimal smooth muscle cells in a coronary artery lesion developing after heart transplantation in man

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Focal intimal thickening is a feature of primary atherosclerotic coronary lesions and restenotic lesions following percutaneous transluminal coronary angioplasty and other forms of vascular intervention, where it is the nonspecific response of the vessel wall to injury. The principal cellular component of the coronary plaque is the smooth muscle cell.¹ Whether the smooth muscle cell in human coronary lesions is derived from cells circulating in the blood or from the vessel wall itself remains a matter for debate. On the basis of animal studies, it is generally presumed that the tunica media or subintimal space is the source of intimal smooth muscle cells.²⁻⁵ However, there is sound experimental evidence that smooth muscle cells recognized in a vascular plaque may originate in mural thrombus and not in the adjacent vessel wall.⁶⁻⁸ Regardless of their source, these cells would normally be indistinguishable. Atherosclerotic lesions developing in the coronary arteries after orthotopic heart transplantation provide a unique opportunity to pursue the origin of cells within the coronary atherosclerotic plaque, as the donor and recipient invariably differ in genotype. Genetic differences may be demonstrated by the electrophoretic analysis of alleles from the highly polymorphic variable number of tandem repeats (VNTR) gene loci that occur widely in the human genome.^{9,10} The D17S5/D17S30 VNTR locus, the D1S80/D1S58 VNTR locus, and the Apo B 3' VNTR locus (the hypervariable 3' region of the apolipoprotein B gene) represent independent, highly polymorphic deoxyribonucleic

acid (DNA) markers that may be used for the purpose of biologic identification.¹¹⁻¹⁵

In this brief report, electrophoretic analysis of alleles from three separate VNTR loci, amplified by the polymerase chain reaction (PCR) technique,¹⁶ was employed to compare the genotype of smooth muscle cells present in a human coronary lesion developing after heart transplantation with that of the immediate postoperative myocardial biopsy and that of recipient leukocytes.

Methods. A 37-year-old man with end-stage heart failure caused by idiopathic dilated cardiomyopathy underwent orthotopic cardiac transplantation. The donor was a 34-year-old woman free of cardiac history. At the conclusion of the transplantation, a baseline myocardial biopsy (biopsy 1) was taken from the right ventricle, placed in isopentane, and frozen immediately under liquid nitrogen (-70° C). Yearly coronary angiograms were performed. After 5 years, a new eccentric stenosis was evident in the circumflex coronary artery (Fig. 1). Exercise thallium 201 perfusion scintigraphy was performed and confirmed a reversible defect in the left ventricular posterior wall. On this basis, the patient underwent directional coronary atherectomy after giving informed consent. The tissue removed from the coronary artery (biopsy 2) was frozen in an identical manner to biopsy 1. Venous blood was drawn from the patient for DNA profiling studies. The presence of smooth muscle cells in the coronary artery lesion was assessed with immunostaining. A portion of the coronary tissue retrieved during directional coronary atherectomy was formalin-fixed and embedded in paraffin. Sections 5 μ m thick were prepared and stained by means of a monoclonal antibody specific for smooth muscle cell α -actin.¹⁷ Genomic DNA was extracted from biopsies 1 and 2 by lysing the tissue in 400 μ l of a solution containing 10 mmol/L tris-HCl (pH 8.0)/10 mmol/L Na₂EDTA/100 mmol/L NaCl/0.5% sodium dodecylsulfate (SDS)/500 μ l proteinase-K in a shaking water bath at 65 $^{\circ}$ C for 4 hours. The lysis mixture was extracted once with tris-saturated phenol, once with chloroform:isoamylalcohol (24:1), and subsequently precipitated in ethanol. The DNA was dissolved in a solution containing 10 mmol/L tris-HCl (pH 8.0)/1 mmol/L Na₂EDTA; DNA from biopsy 1 was dissolved in 15 μ l (final concentration 880 ng \cdot μ l⁻¹) and DNA from biopsy 2 was dissolved in 20 μ l (final concentration 20 ng \cdot μ l⁻¹). To extract genomic DNA from whole blood, the erythrocytes were first lysed by adding 3 volumes of lysis buffer containing 155 mmol/L NH₄Cl/10 mmol/L KHCO₃/1 mmol/L Na₂EDTA and incubating on ice for 30 minutes. After centrifugation, a protocol identical to that detailed for biopsies 1 and 2 was followed for the leukocyte pellet. The final DNA concentration was 800 ng \cdot μ l⁻¹. The yield of genomic DNA from whole blood was 35 μ g \cdot ml⁻¹. For the polymerase chain reaction, genomic DNA (10 ng) was added to a solution containing either 1.0 mmol/L MgCl₂ (D17S5/S30 locus) or 1.5 mmol/L MgCl₂ (D1S80/S58 and Apo B 3' loci) and 50 mmol/L KCl/10 mmol/L tris-HCl (pH 8.3)/200 μ mol/L dNTPs/10% glycerol/0.2 mg \cdot ml⁻¹ acetylated BSA/300 ng primers/1 unit Taq polymerase, with 5% detergent solution. Before temperature cycling, the solutions were

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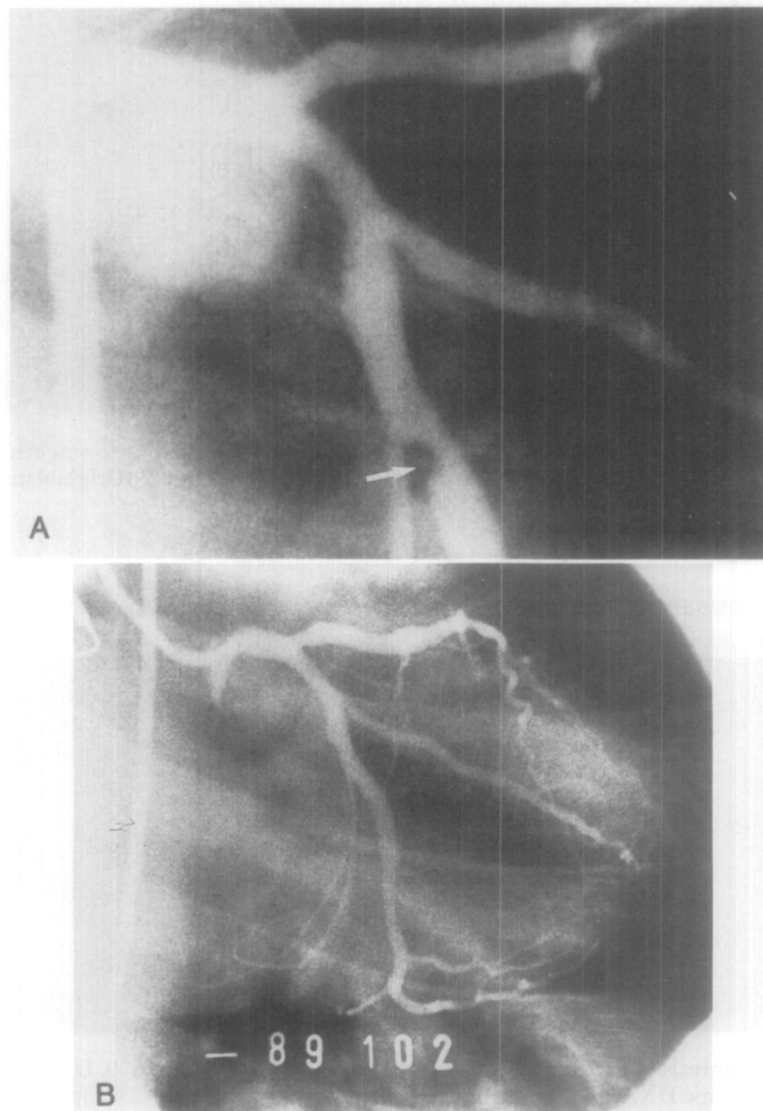


Fig. 1. **A**, Routine coronary angiogram 5 years post-transplant revealing an eccentric stenosis (*arrow*) in the circumflex coronary artery. (Original magnification $\times 4$.) **B**, Control angiogram 1 year previously. No lesion was evident. (Original magnification $\times 1.3$.)

overlaid with 50 μ l mineral oil (Nujol, Plough, Inc., Memphis, Tenn.). After an initial denaturation of 4 minutes at 94° C, 30 cycles of varying incubation time and temperature specific for each locus were performed in a thermal cycler apparatus (Biomed, Germany). Cycle details were the following: D17S5/S30 locus—denaturation 4 minutes at 94° C, annealing 0.5 minutes at 55° C, primer extension 4 minutes at 72° C, final extension 10 minutes at 72° C; D1S80/S58 locus—denaturation 2 minutes at 94° C, annealing 2 minutes at 65° C, primer extension at 4 minutes at 72° C, final primer extension 10 minutes at 72° C; Apo B 3' locus—denaturation 4 minutes at 94° C, annealing 6 minutes at 58° C, primer extension 6 minutes at 58° C, final extension 10 minutes at 58° C. On completion of the polymerase chain reaction (PCR), 5 to 30 μ l aliquots of the

solutions were run on 9% polyacrylamide gel (acrylamide:bisacrylamide, 37:1) in 1 \times TAE (40 mmol/L tris-HCl (pH 7.4)/20 mmol/L sodium acetate/1 mmol/L Na₂EDTA) for 1.5 hours at 200 V in a vertical slab gel electrophoresis apparatus placed in a buffer tank at 50° C.¹⁸ Following electrophoresis, the separation pattern was stained with 30 μ g \cdot ml⁻¹ homidium bromide solution and subsequently documented by Polaroid photography (Polaroid Corp. Medical Products, Cambridge, Mass.) under 302 nm (UVP, Inc., San Gabriel, Calif.). Materials used were monoclonal antibody to smooth muscle cell α -actin (Dako, Copenhagen, Denmark); proteinase-K, glycerol (E. Merck, Darmstadt, Germany); dNTPs (Pharmacia, Uppsala, Sweden); acetylated BSA, detergent, ladder markers (Gibco, Grand Island, N.Y.); and Nujol mineral oil. The polymerase chain

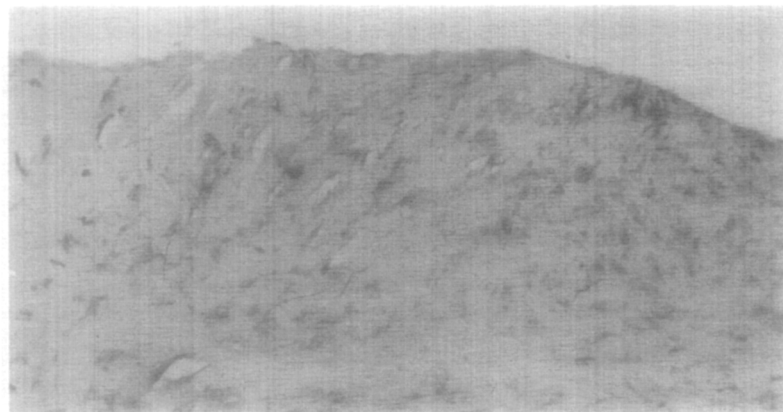


Fig. 2. Coronary atherectomy specimen stained with monoclonal antibodies to smooth muscle cell α -actin. Intimal smooth muscle cells can be identified by their dark pigmentation. (Original magnification $\times 50$.)

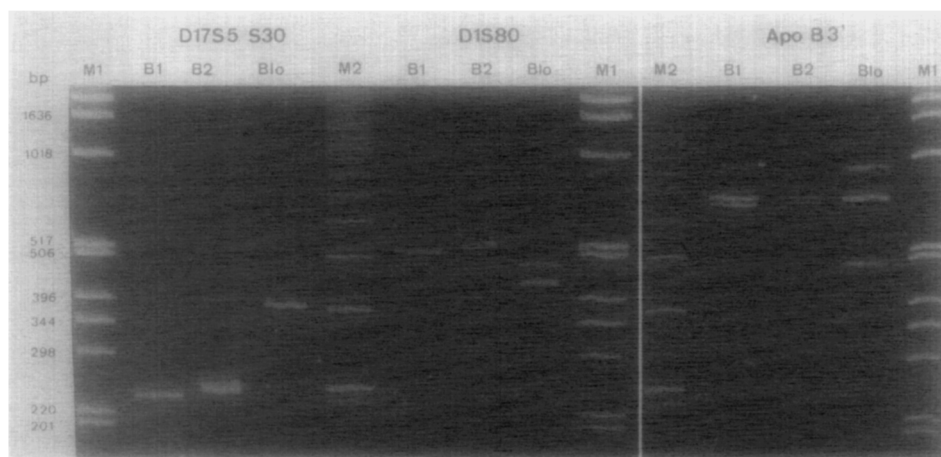


Fig. 3. Gel electrophoresis of products of PCR amplification of the D17S5/S30, D1S80/S58, and Apo B 3' VNTR loci from genomic DNA. *B1* (biopsy 1) is donor myocardial tissue, *B2* (biopsy 2) is coronary tissue retrieved at directional atherectomy, and *Blo* is recipient venous blood. *M1* represents the 1 kb ladder marker, *M2* the 123 bp ladder marker. See text for details.

reaction primer sequences for the three VTNR loci are: D17S5/S30 primer 1—5'-CGAAGAGTGAAGTGCA-CAGG-3' (20 mer) and primer 2—5'-CACAGTCTTTAT-TCTTCAGCG-3' (21 mer); D1S80/S58 primer 1—5'-GAAACTGGCCTCCAAACACTGCCCGCCG-3' (28 mer) and primer 2—5'-GTCTTGTGGAGATGCACGTGC-CCTTGC-3' (29 mer); and Apo B 3' primer 3—5'-CCT-TCTCACTTGGCAAATAC-3' (20 mer).

Results. The tissue obtained at directional coronary atherectomy (biopsy 2) contained cells bearing the morphologic appearance of smooth muscle cells but not arranged with the typical architecture of the tunica media. These cells were identified by immunostaining as smooth muscle cells by their positive staining with anti-smooth muscle cell α -actin (Fig. 2). The result of gel electrophoresis after PCR amplification of alleles of the D17S5/S30, D1S80/S58, and Apo B 3' VNTR loci is shown in Fig. 3. Bi-

opsy 1, biopsy 2, and recipient blood are denoted by B1, B2, and Blo, respectively. M1 and M2 represent the 1 kb and 123 bp ladder markers. For the D17S5/S30 locus (Fig. 3, left panel), PCR products of 372 base pairs (bp) and 550 bp were observed in recipient blood, the latter being only faintly visible because of inefficient amplification of larger alleles of this locus.¹¹ For biopsy 1 and 2, there was a shared fragment of 236 bp, indicating homozygosity of the locus for this allele, and both showed fainter bands corresponding to the 372 bp fragment in recipient blood. This was more evident for biopsy 2. For the D1S80 locus (Fig. 3, center panel), two alleles of 420 bp and 460 bp were observed for recipient blood DNA while the biopsies were homozygous for an additional band of 510 bp. Again, fainter bands corresponding to recipient blood DNA fragments were apparent for the biopsies, more so for biopsy 2. For the Apo B 3' locus (Fig. 3, right panel), three fragments

corresponding to 570, 720, and 880 bp were seen for recipient blood. The 720 and 880 bp fragments also appeared in the biopsies, both of which had a further band of 690 bp. The intensity of the signals suggested that one of the alleles, 720 bp, was shared with blood. The fainter bands corresponding to the blood pattern that were evident for all three loci in the biopsies were further assessed by image analysis of the Polaroid photograph. This indicated that the intensity of the blood-specific bands relative to the biopsy-specific bands for the three loci was approximately 10%.

Comments. With the advent of directional atherectomy, it has become possible to perform biopsies on human coronary plaque tissue and, with advances in molecular biologic techniques, to address fundamental questions related to the development of atherosclerotic lesions. The coronary artery lesion arising *de novo* in the transplanted heart is probably the sole human clinical setting in which the origin, that is, blood-borne versus vessel wall-derived, of smooth muscle cells found in an intimal lesion can be investigated. In this report, the cells within a lesion that developed in the circumflex coronary artery of a transplanted heart were principally of donor genotype, bearing similar genetic features to an immediate post-transplant myocardial biopsy. Thus these cells originated in the vessel wall. However, PCR fragments corresponding to recipient DNA were also identified in the coronary tissue, more apparent for the D17S5/S30 and D1S80/S58 loci. There are two possible explanations. As comparable bands (albeit less intense) were seen in the myocardial biopsy specimen taken directly after transplantation, contamination with recipient blood during removal of the specimens must have occurred. It remains feasible though, that cells derived from circulating recipient blood made up some part of the lesion. For this to be so, in view of the experimental evidence,⁶⁻⁸ previous spontaneous plaque rupture with ensuing mural thrombus deposition would have to be postulated as the setting for the ingrowth of circulatory cells. This is not unreasonable. Plaque rupture is thought to occur commonly in the general population with coronary artery disease,^{19, 20} in whom it need not be associated with symptoms. Indeed, symptoms would not be expected in a heart transplant recipient. In conclusion, the electrophoresis of VNTR loci amplified by the PCR technique indicated that the cells predominant in a *de novo* coronary artery lesion following heart transplantation originated in the vessel wall. Cells of circulatory origin, which contributed as much as 10% of the genetic material associated with the lesion, may have been present as a result of contamination, but we are not entitled to exclude the role of these circulating cells in the development of the lesion.

In summary, the principal cellular component of vascular intimal lesions in primary atherosclerosis and restenosis is the smooth muscle cell. It is believed that these cells originate in the vascular media or subintima, but they may also generate from circulating cells within mural thrombus. We compared the genetic characteristics of cells from a novel coronary artery lesion in a transplanted heart with those of the heart itself (donor genotype) and those of re-

ipient blood leukocytes (recipient genotype). Transplant heart coronary intimal tissue was retrieved at directional atherectomy. Intimal smooth muscle cells were identified by staining with anti-smooth muscle cell α -actin. Electrophoretic analysis of polymorphic VNTR loci, amplified by the PCR technique, was used to compare this tissue with a post-transplant baseline myocardial biopsy and with recipient blood. DNA extracted from intimal cells was predominantly similar to that of the donor myocardium, but features of recipient DNA were also noted in both intima and, to a lesser extent, in myocardium. Smooth muscle cells in a novel coronary artery lesion in a transplanted heart were principally derived from the vessel wall. The presence of cells of circulatory origin may be ascribed to contamination with recipient blood at biopsy, but a contribution of circulating cells to the formation of the lesion is not excluded.

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Morbidity of endomyocardial biopsy in cardiac transplant recipients

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The success rate of cardiac transplantation has greatly improved with the use of the endomyocardial biopsy for monitoring allograft rejection.^{1,2} Although many noninvasive techniques have been used in an attempt to diagnose rejection, endomyocardial biopsy continues to be the gold standard and accepted diagnostic tool in assessment of rejection in the transplanted heart. Two studies have compared the procedural complications of endomyocardial biopsies between heart transplant recipients and nonrecipients with cardiomyopathy. Although endomyocardial biopsy in transplant recipients is generally safe, the incidence of serious complications is 0.3% to 0.7%,³⁻⁵ and there is a definite morbidity and mortality associated with the procedure. This study reviews the procedural complications of 96 orthotopic heart transplant recipients who underwent 1565 endomyocardial biopsy procedures for routine surveillance for cardiac allograft rejection in a single institution.

Records of a consecutive series of heart transplant recipients who underwent endomyocardial biopsy procedures between July 1990 and May 1992 were retrospectively reviewed. All major and minor procedural complications during and after the procedures (an average of 2 hours of observation in the cardiac catheterization laboratory and/or the cardiac transplant clinic) were recorded. The right internal jugular vein was used in 1525 (97.4%) proce-

dures, and the subclavian approach was used in 40 (2.6%) procedures when internal jugular venous access was not obtainable. The Stanford method⁶ for right ventricular endomyocardial biopsy procedure was used. Right heart pressures were measured before and after the biopsy specimens were obtained. All procedures were performed by, or under the direct supervision of one of three attending cardiologists who were experienced in the performance of endomyocardial biopsies. Myocardial tissue samples that were adequate for diagnosis were obtained during all procedures. One thousand five hundred and sixty-two (99.8%) biopsy procedures were performed without complications. There were no major complications (defined as events that resulted in prolongation of hospital stay or death). Three patients experienced the following minor complications (defined as those that could be managed without prolonging the observation time in the cardiac catheterization laboratory and/or clinic). One patient had a 15% pneumothorax, which spontaneously resolved at the time of 1-week follow-up chest roentgenogram. Two patients complained of right shoulder and arm discomfort after the biopsy procedure. One of these patients underwent a neurologic evaluation and was diagnosed with brachial plexus irritation, which resolved with physical therapy. The second patient's discomfort resolved spontaneously in 2 weeks. None of the patients had infection related to biopsy, and no significant arrhythmias such as atrial flutter, atrial fibrillation, or ventricular tachycardia were observed during the procedure.

Endomyocardial biopsy remains the standard for diagnosis of acute allograft rejection in cardiac transplant recipients. Although it is frequently performed and fairly simple, the literature reports a definite morbidity and mortality associated with the procedure. Complications include cardiac perforation, pneumothorax, arrhythmias, neck hematoma, and transient right laryngeal nerve irritations. This study documents that the overall complication rate in cardiac transplant recipients who undergo endomyocardial biopsy is 0.2%. This is a lower incidence than that reported previously in the literature. In summary, endomyocardial biopsy procedure complications are extremely low for experienced operators and can be performed with no mortality and minimal morbidity in cardiac transplant recipients.

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