Human vascular smooth muscle cells of diabetic origin exhibit increased proliferation, adhesion, and migration

Peter L. Faries, MD, Darren I. Rohan, MD, Hidenori Takahara, MD, Mark C. Wyers, MD, Mauricio A. Contreras, MD, William C. Quist, MD, George L. King, MD and **Frank W. LoGerfo, MD,** *Boston, Mass*

Purpose: **Patients with diabetes mellitus (DM) experience progressive macrovascular atherosclerosis and intimal hyperplastic restenosis with increased frequency as compared with nondiabetic patients. These observations suggest that vascular smooth muscle cells (VSMCs) behave in a phenotypically different and more aggressive manner in diabetic patients. In this study, we compared the in vitro rates of proliferation, adhesion, and migration of human VSMCs obtained from diabetic and nondiabetic patients.**

Methods: **Human VSMC cultures were isolated from 23 diabetic patients (9 artery, 14 vein) and 15 nondiabetic patients (9 artery, 6 vein) with extensive lower extremity atherosclerosis. All patients were between 61 and 78 years of age (average: 68.4 years [diabetic]; 67.3 years [nondiabetic]). All diabetic patients had type 2 DM. Vascular specimens were obtained at the time of amputation from infragenicular arteries and during arterial revascularization from saphenous veins. Cells from passages 2 and 3 were assayed for their proliferative capacity with total DNA fluorescence photometry and for adhesion and migration with a modified Boyden chamber.**

Results: **The average duration of diabetes was 11.6 ± 4.1 years. The average number of diabetic complications (retinopathy, neuropathy, nephropathy, coronary artery disease) was 2.8 ± 0.7 per patient. Diabetic VSMCs exhibited abnormal morphology in cell culture with loss of the normal hill and valley configuration. Proliferation was significantly increased in VSMCs of diabetic origin (156 ± 57 absorption units) as compared with those of nondiabetic origin (116 ± 42 absorption units) ($P < .001$). Diabetic VSMCs demonstrated significantly greater adhesion (63.6 ± 24 per high-power field vs 37.9 ± 13 per high-power field; $P = .002$) and migration (397 ± 151 per low-power field vs 121 ± 99 per low-power field; $P = .001$) rates.**

Conclusions: **Diabetic VSMCs exhibit significantly increased rates of proliferation, adhesion, and migration as well as abnormal cell culture morphology suggestive of abnormal contact inhibition. These observations of human VSMCs in culture are consistent with the increased rate of infragenicular atherosclerosis and the increased rates of restenosis observed clinically in diabetic patients. The atherosclerosis- and intimal hyperplasia–promoting behavior exhibited appears to be intrinsic to the DM-VSMC phenotype and must be considered when designing methods to limit atherosclerosis and intimal hyperplasia in diabetic patients. (J Vasc Surg 2001;33:601-7.)**

Diabetes mellitus (DM) affects between 20 and 25 million patients in the United States with an estimated annual cost of more than $50 billion.[1,2] Diabetes has been established as an independent risk factor for atherosclerosis.[3] Patients with diabetes experience progressive macrovascular atherosclerosis, and as a result diabetic patients account for a significant proportion of all peripheral vascular and coronary revascularization procedures.[2,4] In addition, diabetic patients experience greater rates of intimal hyperplasia (IH) as manifested by increased restenosis after coronary angioplasty and increased failure of dialysis access grafts.[5,6] This increased intimal hyperplastic activity may reflect abnormalities of diabetic vascular smooth muscle cells (VSMCs) of venous origin. These observations suggest that VSMCs behave in a phenotypically different and more aggressive proatherogenic manner in patients with diabetes. The purpose of this study was to examine the in vitro rates of proliferation, adhesion, and migration of human VSMCs obtained from diabetic and nondiabetic patients and to determine if differences intrinsic to the DM-VSMC phenotype could be identified.

METHODS

Patients and specimens. Vascular biopsies were obtained at the time of surgery from 38 patients (23 diabetic, 15 nondiabetic). All specimens were obtained under experimental protocol approved by the Institutional Review Board of the Beth Israel Deaconess Medical Center/Harvard Medical School (#98-84E). Arterial vascular specimens were obtained at the time of amputation

From the Division of Vascular Surgery, Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School.

Competition of interest: nil.

Supported by NIH grant R01 HL21796-18.

Presented at the Lifeline Foundation Research Forum of the Joint Annual Meetings of the Society for Vascular Surgery and the American Association for Vascular Surgery, a Chapter of the International Society for Cardiovascular Surgery, Toronto, Ontario, Canada, Jun 11-14, 2000.

Reprint requests: Peter L. Faries, MD, Division of Vascular Surgery, Department of Surgery, Mount Sinai Medical Center, 5 East 98th St, Box 1259, New York, NY 10029 (e-mail: peter.faries@mssm.edu).

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0741-5214/2001/$35.00 + 0 **24/1/111806**

doi:10.1067/mva.2001.111806

Fig 1. Photomicrograph of immunohistochemical preparation of anti–smooth muscle cell α actin–labeled VSMCs in culture. Labeling of actin fibers appears as brown precipitate, confirming that explanted cells are VSMCs in origin.

Vessels of origin of vascular specimens

Site of origin	*Diabetic*	*Nondiabetic*
Infragenicular artery	9	9
Saphenous vein	14	6

from infragenicular arteries. Saphenous vein specimens were obtained during arterial revascularization (Table). VSMCs obtained from nondiabetic patients served as controls for comparison with diabetic VSMCs. All assays were performed on cells derived from distinct, individual specimens. Cell lines were not mixed in the analysis. Clinical patient characteristics, including age, duration of diabetes, insulin requirement, number of secondary complications of diabetes, medical comorbidity, and risk factors for atherosclerosis, were recorded.

VSMC cultures. Cultures were initiated from each specimen immediately after explantation. Primary VSMC cultures were isolated after removal of the adventitia and scraping of the endothelium. Arterial strips were then incubated in Dulbecco modified Eagle medium (DMEM) (Gibco, Life Technologies, Rockville, Md), with 10% fetal bovine serum (FBS) (Gibco), 1% penicillin/streptomycin/amphotericin B (Gibco), and 1 mol/L HEPES (Gibco) at 37°C in 5% carbon dioxide humidified atmosphere. VSMCs were also propagated in DMEM with 10% FBS in subsequent passages. The mean incubation time was 7.3 days. Cell viability was confirmed with trypan blue exclusion after each passage and assay. All assays were conducted with subconfluent cells from passages 2 and 3. VSMC type was confirmed in culture by positive immunolabeling with anti–smooth muscle cell α-actin (mouse monoclonal anti–smooth muscle cell α-actin (clone 1A4) (Neomarkers, Union City, Calif), 1:200 (V/V) with biotinylated horse antimouse immunoglobulin G (H+L) (Vector Laboratories, Burlingame, Calif), 1:100 (V/V) (Fig 1).

Proliferation assay. VSMCs were detached with trypsin-EDTA (Gibco) and replated at a density of 10,000 cells per well. VSMCs were then serum starved in DMEM with 1% FBS for 24 hours to synchronize cell cycles. VSMCs were incubated in DMEM with 10% FBS for 72 hours. The medium was changed daily to maintain appropriate glucose concentration. Cell lysis was performed with 0.05% sodium dodecyl sulfate. DNA was labeled with bisbenzimide (Hoechst, 33258, 1:10,000 (V/V) (Amersham Pharmacia Biotech, Piscataway, NJ). Quantification was performed with a filter fluorescence photometer (TKO 100 fixed excitation bandpass source [365 nm] and emission [460 nm] bandpass filter). This method provides increased accuracy over tritium-thymidine incorporation. Positive controls were calf thymus; negative controls were serum-starved VSMCs, bisbenzimide only. VSMCs from each specimen were assayed six times, and the results were averaged for each specimen individually.

Adhesion assay. After being maintained in DMEM with 1% FBS for 24 hours, VSMCs were detached with trypsin-EDTA (Gibco) and loaded into a modified Boyden adhesion chamber (Osmonics Laboratory and Special Products, Livermore, Calif). A total of 1000 cells were placed into each well that contained a polycarbonate adhesion membrane (Osmonics). The Boyden chamber was incubated at 37°C in a 5% carbon dioxide humidified atmosphere for 2 hours. The adhesion membrane was

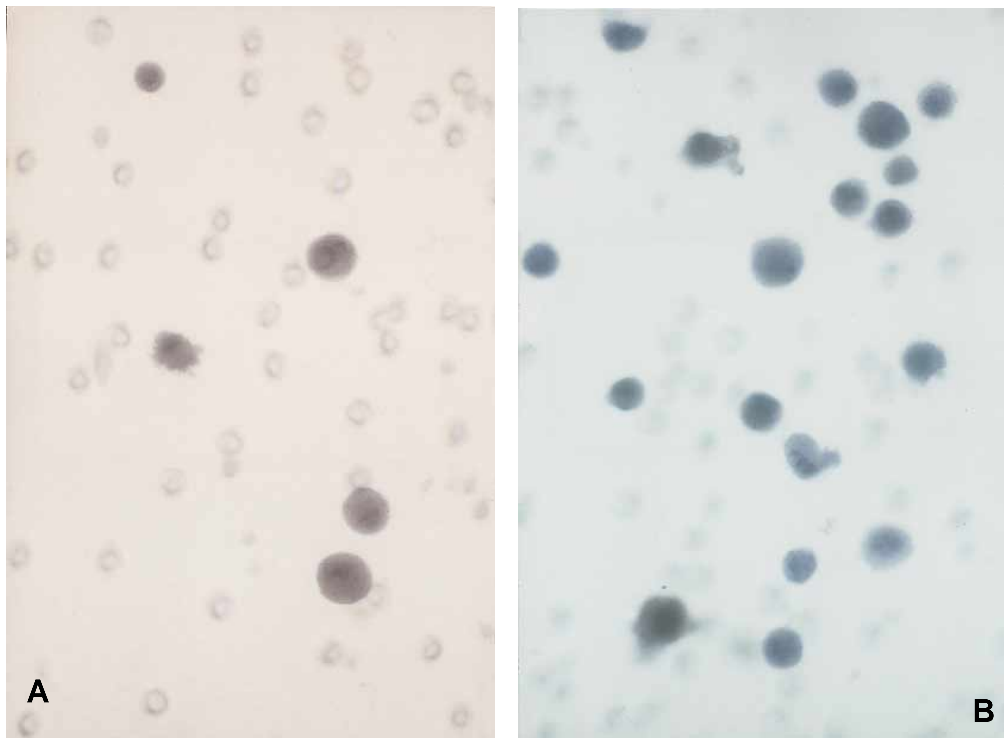

Fig 2. Photomicrograph of VSMCs adherent to polycarbonate adhesion membrane. **A,** Nondiabetic. Relatively few nondiabetic VSMCs are adherent to polycarbonate membrane. **B,** Diabetic. VSMCs of diabetic origin demonstrated adhesion in significantly greater numbers, indicating greater adhesive capacity. VSMCs have been stained with hematoxylin to allow quantification with video image analyzer.

then removed, washed in phosphate-buffered saline, fixed with 90% ethanol, and stained with hematoxylin (Fig 2). Adherent cells were quantified with a digitized video microscopic image analyzer and calibrated image analysis software (Jandel Scientific, Corte Madera, Calif). VSMCs from each specimen were assayed four times, and the results were averaged for each specimen. Adhesion of human VSMCs derived from undiseased aortic cell lines was used for the positive control.

Migration assay. VSMCs assayed for migration were placed in DMEM with 1% FBS for 24 hours. VSMCs were detached with trypsin-EDTA (Gibco) and loaded into a modified Boyden chemotaxis chamber (Corning Costar, Cambridge, Mass). Migration medium containing platelet-derived growth factor–BB as a migratory stimulus at a concentration of 10 ng/mL in DMEM with 10% FBS was loaded into the reservoir that was separated from the VSMCs by the migration membrane (8-μm pore size, 6.5-mm diameter). A total of 10,000 cells were placed in each well, and the chemotaxis chamber was incubated at 37°C in a 5% carbon dioxide humidified atmosphere for 4 hours. After incubation VSMCs were fixed with 70% ethanol and stained with fluorescein isothiocyanate–conjugated nucleic acid stain (Sytox, 1:5000 (V/V), Molecular Probes, Eugene, Ore). Cells that migrated through the membrane were quantified with a digitized video microscopic image analyzer and calibrated image analysis software (Jandel Scientific). Migration of human VSMCs derived from undiseased aortic cell lines was used for the positive control. VSMCs assayed with a migration solution that did not contain platelet-derived growth factor–BB served as the negative control. VSMCs from each specimen were assayed four times, and the results were averaged for each specimen.

Statistical analysis. Continuous variables were compared with a Student *t* test. Statistical significance was assumed at the 95% confidence level ($P < .05$). Data are represented as the mean ± SD.

RESULTS

Patient data. The patients' ages ranged between 61 and 78 years (average: 68.4 years [diabetic]; 67.3 years [nondiabetic]; *P* = not significant). All diabetic patients had type 2 DM. Insulin was required in 85.7%; the remaining 14.3% were non–insulin dependent. Secondary complications of diabetes, including retinopathy, neuropathy, nephropathy, and coronary artery disease, were present in all patients. The average number of diabetic complications was 2.8 per patient (range, 1-4 complications per patient).

Cell culture morphometry. The VSMCs derived from nondiabetic patients demonstrated the normal hill

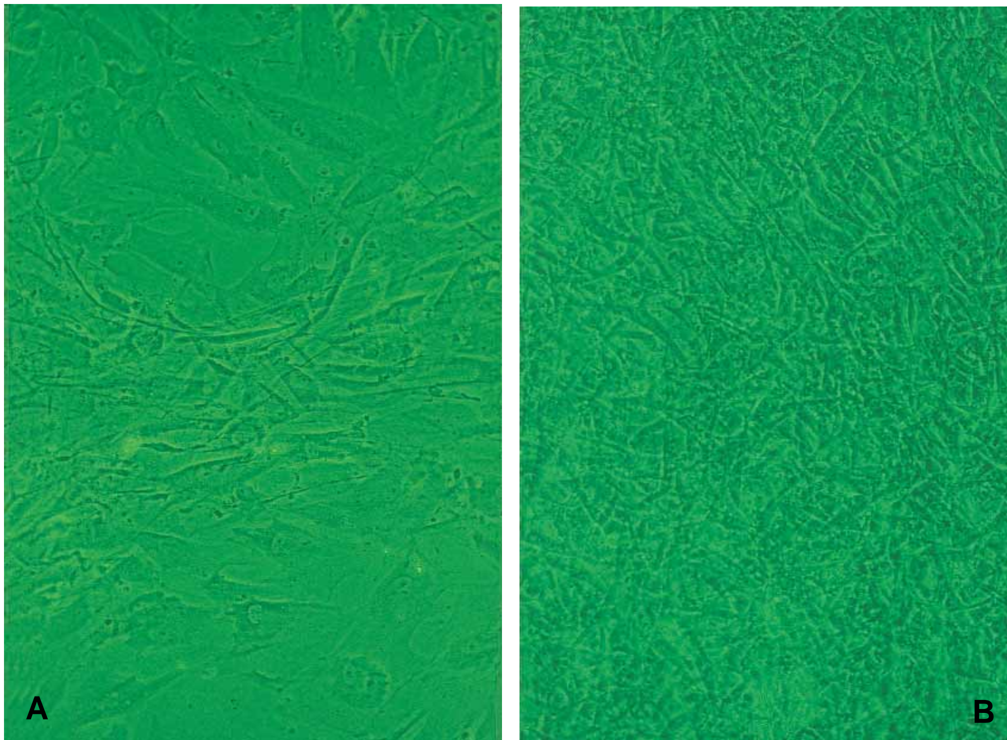

Fig 3. Photomicrograph of VSMCs in cell culture. **A,** Nondiabetic. VSMCs of nondiabetic origin demonstrate the normal hill and valley morphologic appearance in culture, with areas of higher cell density (hill) being separated by areas of low density (valley). **B,** Diabetic. VSMCs of diabetic origin fail to demonstrate normal hill and valley morphology. Diabetic cells are densely packed together with multiple layers of cells superimposed on one another. This appearance suggests loss of normal contact inhibition of proliferation and migration.

and valley morphometry typically observed in VSMCs in culture. In contrast, VSMCs derived from diabetic patients exhibited a loss of the hill and valley morphometry and were densely and continuously packed together in multiple cell layers (Fig 3). The observed loss of normal cell culture morphometry in the VSMCs of diabetic origin suggests a loss of normal contact inhibition of migration and proliferation in these diabetic cells.

Proliferation. Proliferation rates were significantly increased for VSMCs of diabetic origin. Diabetic VSMCs had a mean proliferative rate of 156 ± 57 absorption units as measured with fluorescence photometer. The proliferative rate of VSMC of nondiabetic origin was 116 ± 42 absorption units ($P < .001$; Fig 4).

Adhesion. Adhesion as measured by adherence to a polycarbonate adhesion membrane was significantly increased in VSMCs of diabetic origin as compared with nondiabetic VSMCs. The mean number of diabetic adherent cells was 63.6 ± 24 cells per microscopic field. In contrast, the mean number of adherent cells of nondiabetic origin was 37.9 ± 13 cells per microscopic field ($P = .002$; Fig 5).

Migration. Diabetic VSMCs demonstrated significantly increased migratory capacity in relation to nondiabetic cells. The mean number of cells to migrate in the diabetic cell population was 397 ± 151 cells per microscopic field. This was significantly greater than the rate observed for the nondiabetic cells (121 ± 99 cells per microscopic field, $P = .001$; Fig 6).

DISCUSSION

The increased rate of atherosclerosis and IH experienced by diabetic patients relative to age-matched cohorts of patients without DM suggests an underlying abnormality in the biology of VSMCs in diabetic patients. Evidence of variable phenotypes of VSMCs has been noted by several investigators. The greater incidence of atherosclerotic plaque formation in the abdominal versus the thoracic aorta has been shown with aortic homograft experiments in the canine model to result from differences intrinsic to the VSMC population of those segments of aorta. These phenotypic differences, in turn, are thought to result from distinct lineages of the VSMCs from which the segments of aorta are derived.[7] Variation in VSMC phenotype based on cell lineage has been observed in additional animal models as well.[8] Phenotypic modulation of VSMCs has also been observed in animal models of IH.[7] After exposure to arterial pressure, VSMCs of the conduit vein graft were altered from differentiated, quiescent type to dedif-

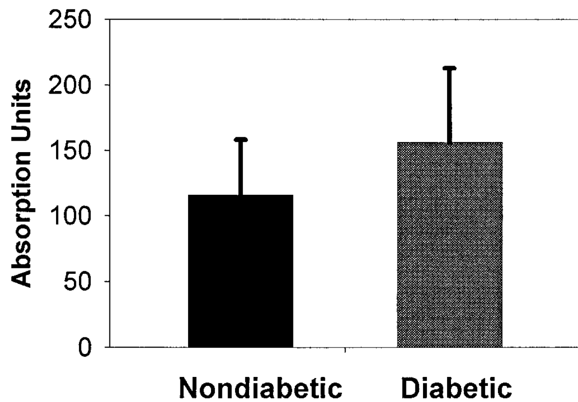

Fig 4. Proliferation—diabetic and nondiabetic VSMCs. VSMCs of diabetic origin demonstrate significantly greater proliferative capacity than do nondiabetic cells ($P < .001$).

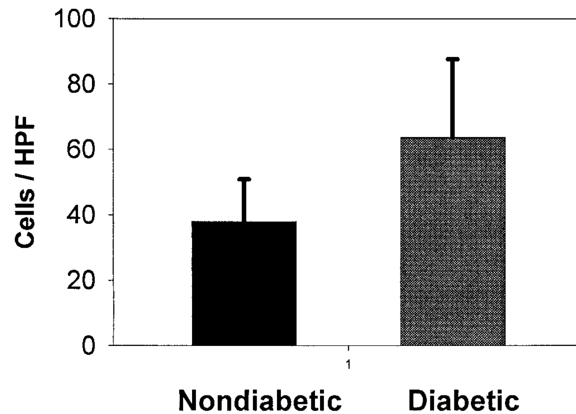

Fig 5. Adhesion—diabetic and nondiabetic VSMCs. VSMCs of diabetic origin demonstrate significantly greater adhesion as compared with nondiabetic VSMCs ($P = .002$).

ferentiated, embryonic, and proliferative type.[9] These observations suggest that variation in VSMC phenotype may result from intrinsic or environmental differences.

Phenotypic variation of VSMCs has also been examined in human-derived cells. In cell culture, VSMCs derived from diseased atherosclerotic tibial arteries have been noted to lose the normal relaxation response to pharmacologic and physiologic stimuli.[10] Altering the culture environment has significant effects on human VSMCs as well. Growth and morphologic characteristics of the VSMCs are altered when they are maintained in coculture with endothelial cells.[11] In addition, the presence of inflammatory cytokines has been shown to induce transformation from the contractile, quiescent VSMC phenotype to the synthetic phenotype in cell culture.[12]

Factors associated with DM have been implicated in altering human VSMC phenotype in cell culture. Both insulin and glucose promote proliferation of VSMCs derived from infragenicular arteries in culture.[13] The proliferative effects of insulin appear to be inhibited by the addition of transforming growth factor–β_1.[14] VSMCs in diabetic resistance vessels have also demonstrated significant abnormalities, including augmented vasoconstrictive response to adrenergic stimuli and impaired relaxation.[15]

Marked phenotypic differences were observed in this study between VSMCs derived from the infragenicular arteries and saphenous veins of diabetic patients and those derived from nondiabetic patients who were matched for degree of atherosclerosis and age, as well as other risk factors for atherosclerosis. These phenotypic differences included increased proliferative capacity, increased cellular adhesion and migration, and the loss of normal cell culture morphology. Cellular proliferation has been widely implicated as being integral to the development of both atherosclerosis and IH.[16-18] A variety of derangements in the control of VSMC proliferation have been identified and associated with increased rates of atherosclerosis and IH.[19]

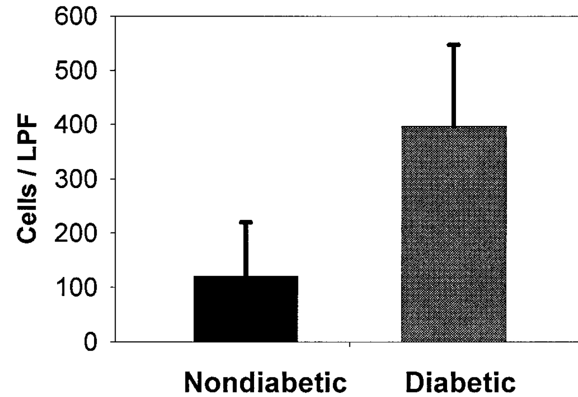

Fig 6. Migration—diabetic and nondiabetic VSMCs. Again, VSMCs of diabetic origin demonstrate significantly greater migration as compared with nondiabetic VSMCs ($P = .001$).

The proliferative rate of diabetic VSMCs in this study was nearly 50% greater than that of nondiabetic VSMCs, a difference that was highly statistically significant. The observed increase in proliferative capacity may well contribute to the development of hyperplastic VSMC lesions in these patients.

The migration of VSMCs from the media to the intima has been emphasized as a necessary event in the development of both IH and atherosclerosis.[20-22] In addition, both overexpression of adhesion molecules in the vessel wall and increased activation of adhesion kinase have been implicated in contributing to the development of atherosclerosis and IH.[23,24] A significant increase in migration and adhesion was observed in VSMCs of the diabetic phenotype in this study. The rate of adhesion for diabetic VSMCs was nearly double that of nondiabetic VSMCs, whereas the rate of migration was more than double for the

diabetic-derived cells. Taken in combination with the significantly increased rate of proliferation, these abnormalities appear to promote proatherogenic activity in the VSMCs and are likely to contribute to the increased rate of atherosclerosis and IH seen in diabetic patients.

Abnormalities of cell culture morphology have been associated with dysregulation of VSMC physiology in previous studies.[11] These abnormalities appear to result, in part, from the loss of normal contact inhibition of proliferation and migration of the VSMCs in culture. While VSMCs derived from nondiabetic arteries and saphenous veins in this study demonstrated the hill and valley configuration normally seen in VSMC cultures, VSMC cultures derived from diabetic patients exhibited loss of the hill and valley morphometry. Instead, the diabetic VSMCs developed multiple layers of densely mixed cells. These observations serve to further highlight the intrinsic abnormalities observed in VSMCs of diabetic origin in this study.

That the differences in proliferation, adhesion, migration, and culture morphometry seen in this study occurred in a constant cell culture environment suggests that the abnormalities are intrinsic to the diabetic VSMC phenotype. These differences are the likely result of long-term exposure to circulating factors in the diabetic patient. Extensive study of the role of advanced glycation end products has been carried out by numerous investigators.[25] It is possible that the accumulation of these advanced glycation end products may promote the proatherogenic activities of the VSMCs seen in this study. In addition, recent investigations have identified abnormalities of 1,2-diacylglycerol levels and the resultant increased activation of protein kinase C that are associated with the development of the secondary vascular complications of diabetes.[26] Abnormally increased diacylglycerol levels and protein kinase C activity may also be contributing to the abnormalities seen in the VSMCs in this study.

In conclusion, VSMCs of diabetic origin exhibit significantly increased rates of proliferation, adhesion, and migration, as well as abnormal cell culture morphology suggestive of abnormal contact inhibition. These observations of human VSMCs in culture are consistent with the increased rate of infragenicular atherosclerosis and increased rates of restenosis observed clinically in diabetic patients. The atherosclerosis- and IH-promoting behavior exhibited appears to be intrinsic to the DM-VSMC phenotype and must be considered when designing methods to limit atherosclerosis and IH in diabetic patients.

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Submitted Jun 13, 2000; accepted Sep 7, 2000.