Inherent differences in morphology, proliferation, and migration in saphenous vein smooth muscle cells cultured from nondiabetic and Type 2 diabetic patients

Haifa A. Madi,¹,* Kirsten Riches,¹,²* Philip Warburton,¹,² David J. O’Regan,²,³ Neil A. Turner,¹,² and Karen E. Porter¹,²

¹Division of Cardiovascular and Neuronal Remodelling, Leeds Institute of Genetics, Health and Therapeutics, University of Leeds, Leeds; ²Multidisciplinary Cardiovascular Research Centre, University of Leeds, Leeds; and ³Department of Cardiac Surgery, The Yorkshire Heart Centre, Leeds General Infirmary, Leeds, United Kingdom

Submitted 26 November 2008; accepted in final form 2 September 2009

Madi HA, Riches K, Warburton P, O’Regan DJ, Turner NA, Porter KE. Inherent differences in morphology, proliferation, and migration in saphenous vein smooth muscle cells cultured from nondiabetic and Type 2 diabetic patients. Am J Physiol Cell Physiol 297: C1307–C1317, 2009. First published September 9, 2009; doi:10.1152/ajpcell.00608.2008.—Individuals with Type 2 diabetes mellitus (T2DM) are at increased risk of saphenous vein (SV) graft stenosis following coronary artery bypass. Graft stenosis is caused by intimal hyperplasia, a pathology characterized by smooth muscle cell (SMC) proliferation and migration. We hypothesized that SV-SMC from T2DM patients were intrinsically more proliferative and migratory than those from nondiabetic individuals. SV-SMC were cultured from nondiabetic and T2DM patients. Cell morphology (light microscopy, immunocytochemistry), S100A4 expression (real-time RT-PCR, immunoblotting), proliferation (cell counting), migration (Boyden chamber assay), and cell signaling (immunoblotting with phosphorylation state-specific antibodies) were studied. SV-SMC from T2DM patients were morphologically distinct from nondiabetic patients and exhibited a predominantly rhomboid phenotype, accompanied by disrupted F-actin cytoskeleton, disorganized α-smooth muscle actin network, and increased focal adhesion formation. However, no differences were observed in expression of the calcium-binding protein S100A4, a marker of rhomboid SMC phenotype, between the two cell populations. T2DM cells were less proliferative in response to fetal calf serum than nondiabetic cells, but both populations had similar proliferative responses to insulin plus PDGF. Under high glucose concentration conditions in the presence of insulin, migration of diabetic SV-SMC was greater than nondiabetic cells. Glucose concentration did not affect SV-SMC proliferation. No differences in insulin or PDGF-induced phosphorylation of ERK-1/2 or components of the Akt pathway (Akt-Ser473, Akt-Thr308, and GSK-3β) were apparent between the two populations. In conclusion, SV-SMC from T2DM patients differ from nondiabetic SV-SMC in that they exhibit a rhomboid phenotype and are more migratory, but less proliferative, in response to serum.

diabetes mellitus; vein graft stenosis; metabolic memory

Diabetes mellitus is a serious and escalating health problem worldwide (3) that currently affects more than 2.1 million people in the United Kingdom, the vast majority of whom have Type 2 diabetes (T2DM) (statistics from Diabetes UK, available at diabetes.org.uk). Diabetes is also associated with increased prevalence of coronary artery disease (CAD) and is an independent risk factor for increased morbidity and mortality (15). Moreover, diabetes is a recognized risk factor for poor outcome following coronary revascularization generally with higher morbidity and short- and long-term mortality (4, 21, 44). Yet up to 25% of patients undergoing coronary revascularization have diabetes, and so understanding the underlying cellular and molecular mechanisms of vein graft stenosis is of major clinical importance (15, 26).

The pathological lesion that underlies vein graft stenosis is intimal hyperplasia (IH), which is defined as excessive smooth muscle cell (SMC) migration and proliferation in the intima of the vein graft wall (23). This is facilitated by the secretion of growth factors, cytokines and matrix metalloproteinases from the endothelium and the SMC themselves, and through the activation of key signaling pathways, including the phosphoinositide 3-kinase (PI3K)/Akt and extracellular signal-regulated kinase (ERK-1/2; also termed p44/42 MAP kinase) pathways (24). During these processes, SMC switch from a quiescent contractile phenotype to an active synthetic phenotype (48) and they deposit extracellular matrix proteins leading to a progressive increase in intimal fibrosis (24). The concept that smooth muscle cells are heterogeneous is widely accepted, and distinct “spindle” and “rhomboid” phenotypes have been identified (6, 17). Rhomboid cells are reportedly more proliferative and migratory than spindle cells and are the more prevalent phenotype in vascular neointimal lesions (16). These rhomboid SMC have recently been characterized as exhibiting elevated expression of the calcium-binding protein, S100A4, which is associated with increased proliferative and migratory activity (6).

Coronary artery bypass grafting (CABG) using the autologous saphenous vein (SV) is commonly used to bypass atherosclerotic vessels. SV grafts of diabetic patients exhibit profound abnormalities of the vessel layers when compared with those of nondiabetic patients, particularly at the intimal level (23). Additionally, diabetic patients tend to have accompanying disorders and more advanced, rapidly progressing disease compared with nondiabetic patients (26). The metabolic effects of hyperglycemia and hyperlipidemia in diabetic patients and their hypercoagulable state may render them vulnerable to vascular complications (13). However, even when blood glucose is well controlled, IH is more prevalent in venous bypass grafts of diabetic subjects, suggesting that additional factors contribute to their poor outcome (26). This raises the question of whether SMC from diabetic patients are intrinsically more “active” than those of nondiabetic patients, a
hypothesis supported by previous studies showing increased proliferation of vascular SMC (VSMC) from diabetic patients compared with nondiabetic patients (11, 27).

To address this, we performed a side-by-side comparison of the in vitro behavior of human SV-SMC from nondiabetic and T2DM patients. Specifically, we investigated their morphology and inherent proliferative and migratory properties to determine whether intrinsic differences exist between SV-SMC from these two patient populations. Our hypothesis was that SV-SMC from T2DM patients would be more proliferative and migratory than their nondiabetic counterparts, thereby contributing to the increased incidence of CAD and the inferior patency of SV bypass grafts.

MATERIALS AND METHODS

Reagents. All cell culture reagents were purchased from Invitrogen, except for fetal calf serum (FCS), which was from Biosera. The manufacturer’s estimate of the insulin content of undiluted FCS was 6–14 µU/ml (<0.12 nM). Culture medium was prepared with a “low” glucose concentration (LG; 5.5 mM) and a “high” glucose concentration (HG; 25 mM). LG was supplemented with mannitol (19.5 mM) to equalize osmolarity of culture media. Platelet-derived growth factor-BB (PDGF), insulin and antibodies for α-smooth muscle actin (α-SMA), smooth muscle myosin heavy chain (SM-MHC), and vinculin were purchased from Sigma. Y27632 was from Calbiochem.

Cell culture. Samples of SV were collected from a total of 22 nondiabetic patients and 22 patients with T2DM undergoing elective CABG surgery at Leeds General Infirmary. Local ethical committee approval and informed, written patient consent were obtained. The mean age of the nondiabetic patients (19 male, 3 female) was 63.3 ± 1.7 yr (range 42–75 yr) and that of the diabetic patients (19 male, 3 female) was 64.8 ± 1.9 yr (range 34–78 yr). Of the diabetic patients, 15 (68%) were receiving oral therapy and 7 (32%) were receiving insulin therapy. SMC were cultured from SV explants as we have described previously (33). Cells were maintained at 37°C in HG Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FCS, in a humidified atmosphere of 5% CO2 in air. All experiments were performed in parallel using SV-SMC of equal passage number [passage 3–5 (P1–P5)] and a similar period in culture, from diabetic and nondiabetic patients. Unless stated otherwise, experiments were performed under HG conditions.

Light microscopy and immunofluorescence. SV-SMC cultures from nondiabetic and T2DM patients were viewed under light microscopy at varying degrees of confluence. For visualization of α-SMA, SM-MHC, F-actin, and vinculin, cells were plated at subconfluent densities onto Lab-Tek chamber slides in full growth medium. After incubation overnight, cells were fixed in 4% paraformaldehyde for 30 min and permeabilized with 1% Triton X-100. Slides were incubated with mouse anti-α-SMA antibody (1:400), mouse anti-SM-MHC (1:200), or mouse anti-vinculin (1:200) at 4°C overnight, followed by FITC-conjugated anti-mouse secondary antibody (1:100) at room temperature for 4 h in the dark. F-actin was visualized by staining with

Fig. 1. Photomicrographs of cultured saphenous vein smooth muscle cells (SV-SMC) from nondiabetic and Type 2 diabetes (T2DM) patients. A and B; low-power (×40) phase-contrast image of SV-SMC in culture. Scale bar: 500 µm. C and D; high-power (×200) phase-contrast image of SV-SMC in culture. Scale bar: 100 µm.
rhodamine-phalloidin (Molecular Probes), as described previously (41). Image analysis was performed using a Zeiss LSM Meta 510 upright confocal microscope.

**Measurement of S100A4 mRNA and protein expression.** For S100A4 mRNA analysis, SV-SMC from six nondiabetic and six T2DM patients were plated at equal density in five culture flasks (1 × 10^5 cells/flask) and cultured for up to 7 days. Cellular RNA was extracted on days 1, 2, 3, 4, and 7 and reverse transcribed as we described previously (43). S100A4 mRNA expression levels were then determined by real-time PCR as previously described (43) using human S100A4 (Hs00243202_m1) and human GAPDH (Hs99999905_m1) primer/Taqman probe sets (Applied Biosystems). S100A4 mRNA levels were calculated as a percentage of GAPDH mRNA levels using the formula 2^(-ΔCT) × 100, in which CT is the threshold cycle number.

For S100A4 protein expression analysis, cells from six nondiabetic and six T2DM patients were cultured for 4 days before preparation of whole cell homogenates. Protein-equalized samples were immunoblotted as we have described previously (42) using S100A4 expression antibody (ab27957, Abcam), and equal loading

Fig. 2. Characterization of human SV-SMC in culture. Representative immunocytochemistry images of human SV-SMC from nondiabetic and T2DM patients following staining with α-smooth muscle actin (α-SMA) and smooth muscle myosin heavy chain (SM-MHC) antibodies. Top: fluorescence. Bottom: phase. All cells expressed both α-SMA and SM-MHC, indicating that cells are exclusively SMC. Scale bar: 100 μm.

Fig. 3. Actin-based cytoskeleton in nondiabetic and T2DM SV-SMC. Rhodamine-phalloidin staining was used to visualize F-actin cytoskeleton. Representative images from cells from different patients are shown. Scale bar: 20 μm: A: SV-SMC from nondiabetic and T2DM patients treated with or without 100 nM insulin for 2 h. B: nondiabetic SV-SMC treated with or without 5 μM Y27632 (Rho kinase inhibitor) for 6 h.
was confirmed with α-tubulin antibody (Abcam). Densitometric analysis was performed using a flat-bed scanner and ImageQuant software (Amersham).

**Proliferation assays.** Cell proliferation assays were performed as described previously (33). Briefly, SV-SMC from one T2DM and one nondiabetic patient (5–6 “pairs” of patients in total) were seeded in parallel into 24-well tissue culture plates at a density of $1 \times 10^4$ cells/well in full growth medium (DMEM plus 10% FCS). Cells were incubated overnight, then quiesced in serum-free medium (SFM) for 3 days before transfer to the appropriate growth-stimulatory medium. This was either full growth medium (10% FCS) or medium containing 0.2% FCS supplemented with PDGF (10 ng/ml) plus insulin (100 nM). Medium was replaced every 2 days, and growth curves constructed over a 7-day period by determining cell number in quadruplicate wells using a hemocytometer. For studies on the effects of glucose concentration, cells were cultured as normal under HG conditions before plating into either LG or HG media 4 days before the start of the experiment.

**Migration assays.** The in vitro migratory properties of SV-SMC from T2DM and nondiabetic patients were assessed using a modified Boyden chamber technique, as we have previously described (33). Serum-deprived cells ($1 \times 10^5$) were loaded in the upper chamber in HG medium supplemented with 0.4% FCS. The lower chamber contained 0.4% FCS (control) or 0.4% FCS supplemented with chemoattractant (10 ng/ml PDGF and 100 nM insulin alone or in combination). In one experimental group, the upper chamber was also supplemented with 100 nM insulin. After incubation for 6 h at 37°C in a tissue culture incubator, duplicate membranes were processed and evaluated by counting cells in 10 random fields under high-power light microscopy (33).

**Signaling experiments.** SV-SMC from T2DM and nondiabetic patients were seeded into six-well plates at equal density ($4 \times 10^4$ cells/well) in full growth medium. After incubation overnight, cells were quiesced in SFM for 2 days before stimulation with 10 ng/ml PDGF and/or 100 nM insulin for 15 min. Whole cell homogenates were prepared and samples were protein equalized and immunoblotted as we described previously (42) using phosphospecific Akt (Ser473), Akt (Thr308), GSK-3β (Tyr204) antibodies (Cell Signaling Technology) or appropriate expression antibodies (all from Cell Signaling Technology except mouse anti-GSK-3β from Santa Cruz Biotechnology). SV-SMC homogenates from both diabetic and nondiabetic patients were analyzed on the same gel and membrane to facilitate accurate comparisons. Densitometry data refer to phosphospecific antibodies and were normalized to the signal in PDGF-treated nondiabetic cells.

**Statistical analysis.** Results are expressed as means ± SE with $n$ representing the number of different patients. Differences between treatment groups were analyzed using paired or unpaired ratio t-tests (GraphPad Prism software) as appropriate. $P < 0.05$ was considered statistically significant.

**RESULTS**

**Cell morphology.** SV-SMC derived from nondiabetic patients clearly exhibited the typical “hill and valley” morphology of SMC in culture (Fig. 1A). On the contrary, SV-SMC derived from T2DM patients appeared dense, broad, and disorganized (Fig. 1B). At high power, nondiabetic SV-SMC were spindle-shaped (Fig. 1C) as opposed to the predominantly rhomboid diabetic SV-SMC (Fig. 1D). Rhomboid morphology was a dependable indicator of diabetic status, accounting for >90% of SV-SMC from T2DM patients. In contrast, SV-SMC of nondiabetic origin were almost always spindle shaped, and cells of rhomboid morphology were infrequent (<5%). Coexpression of α-SMA and SM-MHC confirmed that, despite morphological differences, cells of both nondiabetic and T2DM origin were exclusively SMC (Fig. 2). Fluorescence microscopy following rhodamine-phalloidin staining revealed that SV-SMC of nondiabetic origin had a well-organized actin-based cytoskeleton and long well-aligned F-actin fibers (Fig. 3A). In contrast, T2DM cells exhibited a disorganized actin network with multidirectional fibers of much shorter length (Fig. 3A). Insulin treatment did not visibly modulate the F-actin network in either nondiabetic or T2DM cells (Fig. 3A). When nondiabetic cells were treated with a Rho kinase (ROCK) inhibitor (Y27632), they exhibited actin fiber disruption comparable with that observed in native (untreated) cells from T2DM donors (Fig. 3B).

**S100A4 expression.** Since SV-SMC from T2DM patients appeared to be predominantly of the rhomboid phenotype, we investigated whether S100A4 was expressed at higher levels by cells of diabetic origin than those of nondiabetic origin. Quantitative real-time RT-PCR revealed that while mRNA levels of S100A4 progressively declined (relative to GAPDH) over a 7-day period in both cell populations, there was no significant difference in levels of expression between SV-SMC from T2DM and nondiabetic patients at any time point (Fig. 4A). Immunoblotting revealed that S100A4 protein was expressed...
in SV-SMC from both diabetic and nondiabetic populations (Fig. 4B). S100A4 levels varied considerably between patients, although this was not related to diabetic status (Fig. 4B).

**Proliferation.** Proliferation of SV-SMC from six nondiabetic and six T2DM patients was studied over 7 days in media supplemented with 10% FCS in LG (5.5 mM glucose) and HG (25 mM glucose). After initial plating and serum starving, “day 0” cell counts of diabetic and nondiabetic cells were similar. Proliferation rates were higher for SV-SMC of nondiabetic origin (Fig. 5A). Area under the curve (AUC) analysis revealed that SV-SMC derived from nondiabetic patients had a higher rate of proliferation than those of diabetic origin, either in LG or HG. However, in HG the difference in proliferation between the two cell populations was not statistically significant ($P = 0.121$). Interestingly, glucose had no effect on SV-SMC proliferation independent of diabetic status (Fig. 5A).

To confirm that routine culture of SV-SMC through multiple passages under HG conditions did not induce phenotypic changes, proliferation assays were performed using cells from five further nondiabetic patients that were propagated through P1–P3 in medium containing LG. Proliferation assays performed in parallel under HG and LG conditions yielded comparable cell numbers, indicating that glucose concentration per se was not modulating SMC behavior (Fig. 5C).

To assess whether the inherent difference in proliferative growth between the two populations was due to a specific stimulus, the effect of two well-established key growth factors, PDGF and insulin, was investigated. SV-SMC from nondiabetic and T2DM patients were treated with 0.2% FCS in LG and HG supplemented with PDGF (10 ng/ml) plus insulin (100 nM). In response to PDGF + insulin, proliferation rates were similar for both cell populations (Fig. 5B). This was confirmed by AUC analysis. Once again, glucose concentration had no effect on SV-SMC proliferation (Fig. 5B).

**Migration.** The ability of SV-SMC to migrate toward a PDGF and/or insulin stimulus was analyzed using a modified Boyden chamber assay (Fig. 6). A representative microscopic field of SV-SMC that have migrated to the underside of the membrane in the absence of a chemotactic stimulus (control) or with a PDGF stimulus is shown in Fig. 6A. In nondiabetic cells, insulin induced a 2.2-fold increase ($P = 0.002$) in migration when compared with controls with no chemotactic stimulus (Fig. 6B). In cells from T2DM patients, insulin induced a 3.4-fold increase ($P < 0.0001$) in migration, a response that
experimental groups was not attributable to impaired adhesion to the membranes (data not shown).

In SV-SMC of nondiabetic origin, focal adhesions (characterized by vinculin immunostaining) were infrequent but were commonly observed in T2DM cells and further increased by the addition of insulin (Fig. 7). In addition, there was visible disorganization of the α-SMA network in diabetic SV-SMC compared with the well-aligned filaments in nondiabetic cells (Fig. 8). Interestingly, the addition of insulin appeared to reestablish a degree of organization in the T2DM SV-SMC, with no discernible effect on the nondiabetic cells (Fig. 8).

**Akt and ERK signaling.** SV-SMC from six nondiabetic and six T2DM patients were stimulated with 100 nM insulin, 10 ng/ml PDGF, or both together for 15 min, and activation of the PI3K/Akt and ERK pathways was assessed by immunoblotting with phosphospecific antibodies. Representative immunoblots and pooled densitometry data are depicted in Fig. 9.

Insulin induced a modest (2- to 3-fold) increase in phosphorylation of Akt(Ser473), Akt(Thr308), and GSK-3β in both nondiabetic and T2DM cells (Fig. 9, A–C). PDGF elicited a more robust Akt pathway response, with marked increases in Akt(Ser473), Akt(Thr308), and GSK-3β phosphorylation after 15 min in both cell populations. Neither insulin nor PDGF, alone or in combination, significantly increased ERK-1/2 phosphorylation above basal levels in either cell population (Fig. 9D), most likely due to the high basal activation of this pathway in these cells. When signaling responses in SV-SMC of nondiabetic and T2DM origin were compared (unpaired ratio t-test), no statistically significant differences were observed, although a trend toward increased Akt(Thr308) phosphorylation in response to insulin + PDGF in T2DM cells was observed ($P = 0.063, n = 6$). Expression levels of Akt, GSK-3β, and ERK-1/2 were similar in SV-SMC of nondiabetic and T2DM origin (Fig. 9E).

**DISCUSSION**

The phenotypic heterogeneity of SMC has been well established in humans, as well as many other species. Two morphologically distinct SMC phenotypes have been identified: a spindle-shaped phenotype derived from the normal media with the classical “hill and valley” appearance, and a rhomboid (epithelioid) phenotype, which is reportedly recovered in higher proportions from intimal lesions (5, 16). In the present study, clear differences were noted in the morphology of nondiabetic and T2DM-derived SV-SMC populations. In contrast to nondiabetic SV-SMC, the diabetic cells appeared to lack the typical hill and valley appearance, as has been previously noted (11), and were predominantly of a rhomboid phenotype. Despite the clear morphological differences, coexpression of both α-SMA and SM-MHC in all cell populations confirmed that they were exclusively SMC (29) and not, for example, myofibroblasts. A recent study reported that the calcium-binding protein S100A4 is a marker of rhomboid SMC and is highly expressed in intimal SMC during neointimal development (6). Indeed, S100A4 mRNA levels were reported to be 25.6 times higher in rhomboid SMC than spindle SMC, and S100A4 protein levels were 6 times higher (6). However, when we quantified either S100A4 mRNA or protein levels in our two cell populations, although marked differences were apparent between individuals, these did not appear to be related to
diabetic status per se. Whether the morphology of the diabetic and nondiabetic populations of SV-SMC used in our study is typical of the classical rhomboid and spindle phenotypes therefore requires further clarification. Notably, previous published work was performed using SMC of arterial origin from human and porcine coronary artery (6) and hence might explain our discrepant results. An intriguing observation was that S100A4 mRNA levels in both cell populations were reduced with time in culture, which opposes the theory that S100A4 expression levels are higher in proliferating SMC (6). S100A4 mRNA levels were not influenced by the differing proliferation rates of the two cell populations because data were normalized to GAPDH mRNA levels.

It has previously been reported that arterial SMC of the rhomboid phenotype exhibit higher proliferative and migratory activities than the spindle phenotype (5, 12, 17). In our present study in the presence of insulin, SV-SMC derived from T2DM patients (rhomboid) consistently displayed increased migratory activity although no differences in insulin-induced proliferation rates were apparent. However, in response to stimuli other than insulin (i.e., PDGF and FCS), migration was comparable in both cell populations, although FCS-induced proliferation was significantly lower in diabetic SV-SMC than in nondiabetic cells.

Previous studies have reported that human venous (11) and arterial (27) SMC derived from diabetic patients are more proliferative than those of nondiabetic origin. The discrepancy between our results and these prior studies may relate to the method and/or stimulus used for measuring proliferation, because the former results were obtained by measuring DNA synthesis (11, 27) rather than the more precise method of cell counting that we employed. The reported effects of glucose on proliferation of cultured VSMC are inconsistent. While some studies have shown that high glucose concentration (25 mM) has no effect on proliferation of cultured arterial SMC (39, 47), others report a stimulatory effect (25, 50). In our study the mean proliferation rate of SV-SMC of both cell populations was similar in high and low glucose concentrations, irrespective of the mitogenic stimulus. Our observations are consistent with an earlier study reporting that PDGF-induced VSMC proliferation was similar under low and elevated glucose conditions (39). It is unlikely that the passage number of cells used in our study (P3–P5) could explain either discrepant or comparable results discussed here, since those studies typically used P2–P5 cells (2, 11, 49). One study did, however, report using cells of higher passage (P6–P10) (6).

Since FCS contains a mixture of undefined growth factors, we also assessed the effect of insulin and PDGF-BB, two key mitogens important in neointima development (18). However, in response to insulin plus PDGF, there was no difference in proliferation between the two SV-SMC populations. Earlier investigations using human and primate SMC of both nondiabetic (31, 38) and diabetic (2, 46) origin demonstrated that insulin promoted cell proliferation. Although each of these studies provided evidence that insulin is a potent mitogen for VSMC, ours is the first to directly investigate the effects of insulin on SV-SMC from nondiabetic and diabetic patients in a side-by-side manner.

In the present study, SV-SMC from both nondiabetic and T2DM patients demonstrated a significantly increased migratory capacity when compared with untreated controls, irrespective of the chemotactic stimulus. When insulin alone was used...
as the chemotactic stimulus, a greater increase in SV-SMC migration was observed in the cells of diabetic origin. Intriguingly, in cells of nondiabetic origin, insulin attenuated the migratory response to PDGF, an effect that was not apparent in T2DM cells. These observations concur with a previous study in which insulin inhibited PDGF-induced migration in nondiabetic rat aortic SMC but not in those of Goto-Kakizaki diabetic animals (19). Moreover, in our study these differential effects were still apparent when insulin was included in both the upper and lower wells of the Boyden chamber (i.e., no chemotactic gradient). Thus insulin appeared to have divergent effects on migration of SV-SMC of diabetic and nondiabetic origin independently of its role as a chemotactic stimulus.

In the absence of insulin (i.e., basal or PDGF alone), we saw no difference in migration between the two cell populations, an observation that differs from earlier studies using human SV-SMC (11) or rat aortic SMC (49) in which PDGF-induced migration was higher in cells of diabetic origin. In the human study (11), although PDGF was used as a chemoattractant, the medium also contained 10% FCS, and as such, the migratory stimulus could well have been attributable to any of the undefined growth factors present in FCS.

Vinculin, a component of stable focal adhesions, is associated with increased cell adhesion and stiffness (20) and is also predominantly expressed in rhomboid rather than spindle-shaped cells (22). Our observation of increased vinculin-positive focal adhesions in SV-SMC from T2DM patients may help explain the generalized increased tissue stiffness reported in these patients (45). Vinculin expression is often inversely correlated with migratory capacity (37); however, in our study, cells derived from T2DM patients exhibited both an increase in vinculin-positive focal adhesions and an increase in migration relative to those from nondiabetic patients. Although surprising, this is not without precedent because a recent study also associated a decrease in vinculin-positive focal adhesions with a corresponding decrease in cellular migration (10). Importantly, those data were also derived using human SV-SMC (10). Exposure to insulin has previously been reported to cause an increase in the number of vinculin-positive focal adhesions in human aortic SMC (30), and here we demonstrated that
The α-SMA network is important for cell motility whereby it can act as a positive (40) or negative (34) regulator of migration. We observed a striking disorganization of α-SMA filaments in cells derived from T2DM patients compared with those from nondiabetic patients, and this disorganization was alleviated by the addition of insulin. This may indicate that, in SV-SMC, a structured α-SMA network contributes to enhanced cell migration.

We did not investigate adhesion or apoptosis in our study, although differences have been previously noted. VSMC from patients with diabetes exhibited increased adhesion in vitro compared with those of nondiabetic counterparts (11). The relevance of this functional difference to an in vivo scenario is unclear although the authors speculated that increased cellular adhesion molecules may contribute to the genesis of native atherosclerotic lesions. Upregulation of antiapoptotic genes is also a critical event in vascular lesion formation (32), and hyperglycemia itself has been shown to inhibit VSMC apoptosis in vivo and in vitro (14, 36). Attenuated VSMC apoptosis was observed in diabetic mice (14), and importantly, VSMC cultured from diabetic patients exhibited resistance to apoptosis that was associated with increased levels of the antiapoptotic protein Bcl-2 (35).

Figure 9. Akt and ERK signaling in T2DM and nondiabetic SV-SMC. A–D: SV-SMC from nondiabetic (filled bars) and T2DM (open bars) patients were incubated in the absence (control, C) or presence of 100 nM insulin (I), 10 ng/ml PDGF (P), or both together (I+P) for 15 min before preparation of whole cell homogenates and immunoblotting for phospho-Akt (Ser473) (A), phospho-Akt (Thr308) (B), phospho-GSK-3β (Ser9) (C), or phospho-ERK-1/2 (Thr202/Tyr204) (D). Blots were reprobed with appropriate expression antibodies (total). Densitometry data obtained with phosphospecific antibodies were normalized to PDGF-treated nondiabetic cells. ***P < 0.001, **P < 0.01, *P < 0.05 for effect of treatment vs. appropriate nondiabetic or T2DM control (n = 6, paired ratio t-test). Unpaired ratio t-tests comparing nondiabetic with diabetic signaling responses were all not statistically significant (n = 6). E: densitometric analysis of total Akt, GSK-3β, and ERK-1/2 expression in nondiabetic (filled bars) and T2DM (open bars) patients.
(IMA) function revealed that IMA from T2DM patients exhibited reduced Akt (Thr308) phosphorylation (28) and increased ERK-1/2 and p38-MAPK phosphorylation (8) compared with IMA from nondiabetic patients. In our study using cultured SV-SMC we observed no significant differences in phosphorylation of Akt (Ser473 and Thr308), GSK-3β, or ERK-1/2 between cells of T2DM or nondiabetic origin in response to insulin or PDGF. Our investigation was, however, limited to studying only a single time point of activation (15 min) and did not explore the full time course of signaling responses. Whether differences in these signaling pathways were apparent at the time of tissue collection was not determined. Nevertheless, differences in Akt or ERK-1/2 signaling do not appear to explain the differential functional responses (proliferation, migration) observed in SV-SMC of T2DM and nondiabetic origin. However, we did observe that SV-SMC of diabetic origin possessed short, multidirectional F-actin fibers, a condition that could be mimicked by ROCK inhibition in nondiabetic cells. It is possible that aberrancies of the Rho/ROCK pathway in cells from T2DM patients may provide a mechanism for the altered morphology and migration in the T2DM group, and further studies are warranted to substantiate this hypothesis.

A key strength of our study was the use of human SV-SMC from several different nondiabetic and T2DM patients. These cells are directly responsible for the development of intimal hyperplasia in SV bypass grafts in vivo. An obvious limitation of this study was its in vitro nature, which does not take into account all the variables encountered in the in vivo situation, such as blood flow, pressure, metabolic factors, and the influence of the vascular endothelium. However, the use of SV-SMC in isolation provides a model that permits specific stimuli to be investigated in the absence of these confounding variables. Another potential limitation was the use of 100 nM insulin, a level that is ~300-fold higher than would be achieved in vivo after a meal in normal subjects (~0.3–0.4 nM) or ~70-fold higher than in insulin-resistant patients (~1.4–1.5 nM). We designed our study to compare with the literature in which the use of supraphysiological concentrations in vitro is routine. This is perhaps not surprising given the myriad differences between in vitro functional assays and the in vivo scenario, not least the differences in time scales. It is, however, noteworthy that a lower concentration of insulin (10 nM) could also induce marked Akt signaling and increased cell proliferation in SV-SMC, although the magnitude of the effect was lower (data not shown). Future studies should compare more physiological and pathophysiologically relevant insulin concentrations.

In conclusion, our study has revealed a number of key findings. First, we have shown that SV-SMC of nondiabetic origin were generally of a spindle phenotype, whereas those from T2DM patients appeared rhomboid; however, this did not correlate with S100A4 expression. Second, SV-SMC from T2DM patients exhibited a lower rate of proliferation in response to FCS, and a higher migration rate in the presence of insulin compared with cells from nondiabetic patients. Disparate focal adhesion formation and β-actin organization in response to insulin (possibly due to altered Rho/ROCK signaling) may account, at least in part, for these differences. Third, glucose concentration did not modulate the rate of SV-SMC proliferation irrespective of mitogenic stimulus or diabetic status. Finally, there were no significant differences in Akt and ERK signaling responses in T2DM and nondiabetic cells. Our study may provide evidence of “metabolic memory” (7), the long-term molecular and cellular changes that are a consequence of hyperglycemia. Given the complexity of the diabetic milieu, continued detailed investigation of the cellular and molecular mechanisms underlying the vascular complications of T2DM is warranted.

ACKNOWLEDGMENTS

We are grateful to Jean Kaye and Stacey Galloway for cell culture expertise and to Dr. Gareth Howell (Faculty of Biological Sciences, University of Leeds) for expert assistance with confocal microscopy.

GRANTS

H. A. Madi was supported by a Heart Research UK Intercaled Scholarship (RG2525/06/07). N. A. Turner is in receipt of a Research Councils UK Academic Fellowship.

REFERENCES


