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Type 2 diabetes impairs venous, but not arterial smooth muscle cell function: Possible role of differential RhoA activity

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ABSTRACT

Background/purpose: Coronary heart disease is the leading cause of morbidity in patients with type 2 diabetes mellitus (T2DM), frequently resulting in a requirement for coronary revascularization using the internal mammary artery (IMA) or saphenous vein (SV). Patency rates of SV grafts are inferior to IMA and further impaired by T2DM whilst IMA patencies appear similar in both populations. Smooth muscle cells (SMC) play a pivotal role in graft integration; we therefore examined the phenotype and proliferative function of IMA- and SV-SMC isolated from non-diabetic (ND) patients or those diagnosed with T2DM.
Methods/materials: SMC were cultured from fragments of SV or IMA. Morphology was analyzed under light microscopy (spread cell area measurements) and confocal microscopy (F-actin staining). Proliferation was analyzed by cell counting. Levels of RhoA mRNA, protein and activity were measured by real-time RT-PCR, western blotting and G-LISA respectively.
Results: IMA-SMC from T2DM and ND patients were indistinguishable in both morphology and function. By comparison, SV-SMC from T2DM patients exhibited significantly larger spread cell areas (1.5-fold increase, $P < 0.05$), truncated F-actin fibers and reduced proliferation (33% reduction, $P < 0.05$). Furthermore, lower expression and activity of RhoA were observed in SV-SMC of T2DM patients (37% reduction in expression, $P < 0.05$ and 43% reduction in activity, $P < 0.01$).
Conclusions: IMA-SMC appear impervious to phenotypic modulation by T2DM. In contrast, SV-SMC from T2DM patients exhibit phenotypic and functional changes accompanied by reduced RhoA activity. These aberrancies may be epigenetic in nature, compromising SMC plasticity and SV graft adaptation in T2DM patients.
Summary: The internal mammary artery (IMA) is the conduit of choice for bypass grafting and is generally successful in all patients, including those with type 2 diabetes (T2DM). By contrast, saphenous vein (SV) is inferior to IMA and furthermore patients with T2DM suffer strikingly poorer outcomes than their non-diabetic (ND) counterparts. We discovered that SV-SMC from T2DM patients exhibit altered persistent morphology and function compared to ND SV-SMC, with differential expression and activity of the small GTPase RhoA, yet ND and T2DM IMA-SMC were indistinguishable. These data offer an explanation for the superior patency of IMA grafting independent of the presence of diabetes.

1. Introduction

Type 2 diabetes mellitus (T2DM) is an escalating global epidemic, and in the UK alone the number of patients with diagnosed T2DM has almost doubled over the past 15 years (diabetes.org.uk). Importantly, treatment of patients with T2DM and its resultant complications now accounts for approximately 10% of the entire UK National Health Service budget. One of the leading causes of morbidity and mortality in patients with T2DM is accelerated atherosclerosis and coronary heart disease [1] that often precedes clinical diagnosis of T2DM [2].

The surgical approach to revascularizing atherosclerotic coronary arteries is coronary artery bypass grafting (CABG) using autologous internal mammary artery (IMA) or saphenous vein (SV) to restore blood supply to the ischemic heart. Whilst the IMA is known to be a superior conduit with patency rates significantly higher than SV [3], due to its limited availability and the need for multiple grafts the SV is routinely the conduit of choice in many patients. Furthermore, patients with T2DM have poorer SV graft outcomes compared to their non-diabetic counterparts [4]. Interestingly, this is not the case with IMA, in which patency rates are comparable between patients with or without T2DM (reviewed recently in [5]).

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IMA and SV are structurally distinct vessels [3] that respond differently to alterations in pressure and cyclical stretching that are evident when vessels are implanted following CABG. This leads to changes in vessel structure and function, e.g. altered distensibility and stiffness (reviewed in [6]) and intimal abnormalities [7], due at least in part to the orientation and behaviour of smooth muscle cells (SMC). Successful integration of grafts early after implantation requires efficient adaptive remodeling [8], a process that involves phenotypic switching of SMC in terms of co-ordinated migration, proliferation and cytoskeletal rearrangement. This functional capacity to adapt is temporally distinct from the subsequent neointimal thickening that underlies narrowing and restenosis. The signaling cascades regulating these processes are complex and include mitogen activated protein kinases (MAPK) such as extracellular signal regulated kinase (ERK) and p38 MAPK [9], amongst others. Small GTPases and in particular RhoA/Rho kinase are also well recognized effectors of such adaptive changes [10].

RhoA is an archetypal member of the Rho family of small GTPases, activation of which promotes formation of F-actin stress fibers and focal adhesions which link stress fibers to the plasma membrane, thereby affecting SMC contractility and adhesion [11]. RhoA regulates many cellular functions including migration and proliferation [11], dysregulation of which are implicated in cardiovascular disorders such as hypertension, coronary artery vasospasm and neointimal hyperplasia (reviewed in [10]). RhoA is reportedly activated by hyperglycemia, and accordingly aberrant RhoA activity has been demonstrated in rodent models of diabetic nephropathy [12] and myocardial fibrosis [13].

We have previously reported inherent differences in the morphology and function of SV-SMC from non-diabetic (ND) and T2DM patients; specifically that SV-SMC from patients with diabetes exhibited rhomboid-like morphology, altered cytoskeletal arrangement and impaired proliferative capacity compared to those isolated from patients without diabetes [14]. Of particular interest was our observation that the cellular disparities were maintained throughout culture and serial passaging and not influenced by glucose concentration. We therefore speculated that through prior exposure to the metabolic milieu, SMC from diabetic patients show evidence of "memory". The aim of this study was therefore to investigate any influence of T2DM on IMA-SMC phenotype and to determine a potential role for RhoA.

2. Materials and methods

2.1. SMC isolation and culture

SMC were obtained from IMA and SV fragments from a total of 63 different patients undergoing CABG at Leeds General Infirmary, UK, and were cultured by explant technique as described previously [15]. Local ethical committee approval and informed patient consent were obtained. This study conformed to the principles outlined in the Declaration of Helsinki. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1% L-Glutamine and 1% penicillin/streptomycin/fungizone (full growth media – FGM) at 37 °C in 5% CO_2 in air. SMC were serially passaged using trypsin/EDTA as necessary. All cells were isolated and sub-cultured for up to 6 passages; individual experiments were performed on SMC of equivalent passage from age-matched donors with diagnosed T2DM or without (ND), characteristics of whom are documented in Table 1. All investigations were performed on cells between passages 2 and 6, an interval over which we have previously identified a stable phenotype [14].

2.2. Cell area measurements

SMC were seeded at a density of 2×10^5 cells per 75 cm^2 flask, cultured for 96 h and then examined under phase contrast microscopy at ×100 magnification. Fifteen random fields of view were captured and the spread areas of 50 cells per patient were measured using ImageJ software (http://imagej.nih.gov/ij/). In addition to calculating the average cellular area per patient population, data were used to record the distribution of cell sizes (5000 μm^2 increments) within each population.

Table 1
Donor patient characteristics.

		Mean Age (Range)	Sex	T2DM Treatment
IMA-SMC ND	n = 19	64.5 years (49–82)	89% Male	N/A
IMA-SMC T2DM	n = 16	61.8 years (34–82)	94% Male	12.4% diet controlled 43.8% oral therapy 43.8% oral plus insulin therapy
SV-SMC ND	n = 25	64.8 years (49–82)	92% Male	N/A
SV-SMC T2DM	n = 23	61.4 years (34–85)	100% Male	4.3% diet-controlled 43.5% oral therapy 52.2% oral plus insulin therapy

All SMC groups were from age-matched donor patients, predominantly (>90%) male. The majority of T2DM patients were receiving oral or oral plus insulin therapies and a smaller percentage were on diet management programmes.

2.3. Immunocytochemistry

SMC were seeded at a density of 2×10^3 per well in Lab-Tek chamber slides, cultured for 96 h in FGM and then fixed in 4% paraformaldehyde. Immunostaining for smooth muscle myosin heavy chain (SM-MHC; AbCam 1:100), alpha smooth muscle actin (α-SMA; Sigma-Aldrich 1:200), F-actin (Invitrogen, 1:50) and vinculin (Sigma-Aldrich, 1:400) was performed essentially as previously described [14]. Images were visualized using a Zeiss LSM 510 confocal microscope at ×400 magnification.

2.4. Proliferation assays

Proliferation assays were performed essentially as we described previously [14]. Briefly, cells were seeded at 1×10^4 cells per ml in 24-well plates, cultured for 24 h, then quiesced in serum-free medium for 72 h prior to making triplicate 'day 0' counts using trypan blue and a hemocytometer. SMC were maintained in FGM for up to 7 d. Media were replenished on days 2 and 4 and triplicate viable cell counts taken on days 2, 4 and 7 to generate a growth curve from which area under curve (AUC) analysis was performed.

2.5. RhoA real-time RT-PCR

SMC were seeded at a density of 2×10^5 in 25 cm^2 flasks and cultured in FGM for 96 h before total mRNA was isolated as previously described [16]. Real-time reverse transcription PCR was used to determine RhoA mRNA expression using specific TaqMan assays (Life Technologies, Hs00357608_m1). RhoA expression was calculated as a percentage of GAPDH (Hs99999905_m1) using the formula $2^{-\Delta CT} \times 100$.

2.6. RhoA Western blotting

Reduced whole cell lysates were prepared and immunoblotted for RhoA (Santa Cruz, 1:100) or GAPDH (Sigma-Aldrich, 1:4000) as previously described [17]. Expression of RhoA and GAPDH was normalized to a single sample that was included on all membranes to allow comparison between experiments.