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Investigating inherent functional differences between human cardiac fibroblasts cultured from nondiabetic and Type 2 diabetic donors

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ABSTRACT

Introduction: Type 2 diabetes mellitus (T2DM) promotes adverse myocardial remodeling and increased risk of heart failure; effects that can occur independently of hypertension or coronary artery disease. As cardiac fibroblasts (CFs) are key effectors of myocardial remodeling, we investigated whether inherent phenotypic differences exist in CF derived from T2DM donors compared with cells from nondiabetic (ND) donors.
Methods: Cell morphology (cell area), proliferation (cell counting over 7-day period), insulin signaling [phospho-Akt and phospho-extracellular signal-regulated kinase (ERK) Western blotting], and mRNA expression of key remodeling genes [real-time reverse transcription-polymerase chain reaction (RT-PCR)] were compared in CF cultured from atrial tissue from 14 ND and 12 T2DM donors undergoing elective coronary artery bypass surgery.
Results: The major finding was that Type I collagen (COL1A1) mRNA levels were significantly elevated by twofold in cells derived from T2DM donors compared with those from ND donors; changes reflected at the protein level. T2DM cells had similar proliferation rates but a greater variation in cell size and a trend towards increased cell area compared with ND cells. Insulin-induced Akt and ERK phosphorylation were similar in the two cohorts of cells.
Conclusion: CF from T2DM individuals possess an inherent profibrotic phenotype that may help to explain the augmented cardiac fibrosis observed in diabetic patients.
Mini Summary: We investigated whether inherent phenotypic differences exist between CF cultured from donors with or without Type 2 diabetes. Cell morphology, proliferation, insulin signaling, and gene expression were compared between multiple cell populations. The major finding was that Type I collagen levels were elevated in fibroblasts from diabetic donors, which may help explain the augmented cardiac fibrosis observed with diabetes.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disease associated with sedentary lifestyles and obesity which manifests as a result of insulin resistance. Currently T2DM affects over 250 million people worldwide, and its prevalence is predicted to rise significantly over the coming years [1]. Overt T2DM is often preceded by several years of insulin resistance during which time the pancreas maintains glycemic control by secreting increased levels of insulin. Consequently, diagnosis of T2DM is often made only when pancreatic insulin secretion becomes insufficient to maintain normoglycemia and symptoms of hyperglycemia ensue. Diabetic patients therefore experience long-term metabolic disturbances that can have detrimental effects well before diagnosis. T2DM manifests with a heightened inflammatory and profibrotic state [2,3] and is an independent risk factor for cardiovascular disease in both men and women [4]. The association between T2DM and heart failure occurs irrespective of etiology (ischemic or nonischemic) [5], suggesting that T2DM confers detrimental effects directly on the heart. Indeed, the term *diabetic cardiomyopathy* is used to describe a pathology that occurs independently of coronary artery disease and hypertension and manifests as increased left ventricular mass and reduced ventricular contractility, together with interstitial fibrosis and increased diastolic stiffness [6,7]. Although often manifesting as changes in ventricular remodeling, T2DM is also strongly associated with atrial fibrillation [8] which can drive subsequent atrial remodeling [9]. The cellular and molecular mechanisms underlying the heightened inflammatory and fibrotic states associated with T2DM are yet to be fully elucidated.

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Cardiac fibroblasts (CF) [10] are the most prevalent cell type in the heart. In health they are relatively quiescent cells responsible for maintaining cardiac extracellular matrix (ECM) homeostasis through regulated collagen and matrix metalloproteinase (MMP) synthesis [10]. In cardiovascular disease, increased mechanical wall stress and biochemical stimuli induce phenotypic differentiation of CF into hypersecretory myofibroblast cells [10–12]. Fibroblasts and myofibroblasts regulate many aspects of cardiac pathophysiology, and as well as being important for the repair of the heart after injury (e.g., myocardial infarction) they also contribute to fibrosis and heart failure progression [10–12].

Despite the significance of CF in regulating cardiac remodeling there is relatively little known about whether diabetes can directly modulate CF function. There are several reports describing in vitro effects of elevated glucose concentration on CF function [13–16]; however, hyperglycemia represents only one component of the complex diabetic milieu. Very recent evidence from rat and mouse models of T2DM has suggested that there are functional differences in CF derived from T2DM hearts compared with control hearts [17,18]; however, there is no current evidence that such differences exist in human CF.

The aim of the present study was to determine whether there are phenotypic differences in cultured CF derived from T2DM patients compared with cells from donors without diagnosed diabetes; differences that may contribute to adverse cardiac remodeling in T2DM individuals. Accordingly, we compared cell morphology, cell proliferation, insulin signaling, and specific gene expression responses in CF derived from a cohort of 14 nondiabetic (ND) and 12 T2DM donors.

2. Materials and methods

2.1. Cell culture

CF were isolated and cultured as described previously [19–21] by collagenase digestion of right atrial appendage samples from 26 patients without left ventricular dysfunction undergoing coronary artery bypass graft surgery at Leeds General Infirmary. Local ethical committee approval and informed patient consent were obtained. CF were cultured in full growth medium (FGM) comprising Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum (FCS), 100-μg/ml penicillin-streptomycin, and 2-mM L-glutamine, as described previously [19–21]. Experiments were performed on CF from passages 3–6. Experiments were conducted on cells from a total cohort of 14 ND Ctrl donors (86% male, mean age: 64.8±1.7, range: 50–75) and 12 T2DM donors (92% male, mean age: 68.2±3.1, range: 42–83). Of the diabetic individuals, 5 were receiving oral therapy, 5 were receiving insulin plus oral therapy, and 2 were diet-controlled.

2.2. Cell area calculation

Images were taken of subconfluent cell populations from individual ND and T2DM donors (10 patients per cohort) at ×100 magnification under light microscopy using Ulead Photo Explorer 7.0. Outlines of the first 50 cells per donor were drawn using Image J software (http://imagej.nih.gov/ij) and cell areas calculated and averaged from the number of encompassed pixels before conversion to μm^2.

2.3. Proliferation assays

Proliferation assays were conducted as described previously [20]. Briefly, cells from ND and T2DM donors (eight patients per cohort) were plated at 1×10^4 cells per well in 1-ml FGM and incubated overnight prior to being growth arrested in serum-free medium (SFM) for 72 h. FGM was refreshed on Days 0, 2, 4, and 7, and quadruplicate cell counts were conducted using a hemocytometer and Trypan Blue.

2.4. Western blotting

Serum-starved cells from 5 ND and 5 T2DM donors were incubated with 100-nM insulin for 5–60 min before preparing whole-cell homogenates by scraping the cell layer into sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer supplemented with phosphatase and protease inhibitors [22]. Equal protein concentrations of samples (typically 10 μg/lane) were resolved by SDS-PAGE prior to Western blotting with primary antibodies (Cell Signaling Technology) for phospho-Akt(Ser-473) or phospho-ERK(Thr-202/Tyr-204) [22]. Densitometric analysis was performed using Image J software and data expressed relative to a loading control included on each gel.

For assessment of Type I collagen (COL1A1) protein levels, equal protein concentrations (10 μg) of control samples from signaling experiments were resolved on the same gel before probing membranes with anti-COL1A1 antibody (sc-8784; Santa Cruz Biotechnology). Membranes were reprobed with anti-β-actin antibody (ab8226; Abcam) as a loading control, and densitometric data expressed as the ratio of COL1A1 to β-actin levels.

2.5. Quantitative real time-polymerase chain reaction (PCR)

Serum-starved cells from 6 ND and 6 T2DM donors were incubated with low-serum medium (SFM supplemented with 0.4% FCS) containing 10-ng/ml interleukin (IL)-1α for 6–24 h. This concentration of IL-1α was selected based on our previous study of cytokine gene expression in human CF [23]. RNA extraction and reverse transcription were performed as described previously [24]. Real-time PCR was performed in triplicate using the Applied Biosystems ABI-7500 System and Taqman primer/probes for IL1β (Hs00174097_m1 primer/probe set), IL6 (Hs00174131_m1), IL8 (Hs99999034_m1), COL1A1 (Hs00164004_m1), ACTA2 (Hs00426835_g1), MMP2 (Hs01548727_m1), and MMP3 (Hs00233962_m1). Data are expressed relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression (Hs99999905_m1) using the formula $2^{-\Delta CT}\times100$ [24].

2.6. Statistical analysis

Results are presented as mean±standard error, and the number of experiments on cells from different patients is represented by *n*. Data were analyzed with GraphPad Prism 6 software using Student *t* tests or two-way analysis of variance (ANOVA) with Holm–Sidak's post-hoc test, as appropriate. $P<.05$ was considered statistically significant.

3. Results

3.1. Influence of T2DM on cell morphology

We investigated whether there was an association between diabetic status and cellular morphology in CF cultured from multiple ND and T2DM donors. Mean spread cell areas were calculated from 50 cells per patient, with representative images shown in Fig. 1A. The combined data showed that there was more variation in cell size in the T2DM group compared with ND controls (Fig. 1B). Moreover, there was a strong trend toward increased cell size in cells from T2DM donors compared with ND (Fig. 1B), although this did not quite reach statistical significance ($P=.079$).

3.2. Influence of T2DM on proliferation rate

Cell proliferation profiles for CF from eight ND and eight T2DM donors were constructed by culturing cells in FGM and counting cells over a 7-day period (Fig. 1C). A 3.3-fold and 2.7-fold increase in cell