ErbB2 Expression on Left Ventricular Epicardial Endothelial Cells and CD105+ Cells is Decreased in Patients with Diabetes Mellitus.

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Abstract

Background

We investigated the cell surface expression of ErbB receptors on left ventricular (LV) epicardial endothelial cells and $CD105^+$ cells obtained from cardiac biopsies of patients undergoing coronary artery bypass grafting surgery (CABG).

Methods

Endothelial cells and $CD105^+$ non-endothelial cells were freshly isolated from LV epicardial biopsies obtained from 15 subjects with diabetes mellitus (DM) and 8 controls. The expression of ErbB recepotrs was examined using multiparametric flow cytometry. Human microvascular endothelial cells (HMEC-1) and LV epicardial $CD105^+$ non-endothelial cells were used to determine the effect of high glucose on ADAM10-dependent cleavage of ErbB receptors.

Results

We found that diabetes mellitus (DM) and high levels of hemoglobin A1C are associated with reduced expression of ErbB2 on both endothelial cells and $CD105^+$ non-endothelial cells. To determine if the expression of ErbB2 receptors is regulated by glucose levels, we examined the effect of high glucose in HMEC-1 and LV epicardial $CD105^+$ non-endothelial cells, using a novel flow cytometric approach to simultaneously determine the total level, cell surface expression, and phosphorylation of ErbB2. Incubation of cells in the presence of 25 mM D-glucose resulted in decreased cell surface expression of ErbB2. We also found high expression of a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) on both endothelial cells and $CD105^+$ non-endothelial cells. Inhibition of ADAM10 prevented the high glucose-dependent decrease in the cell surface expression of ErbB2.

Conclusions

We suggest that high glucose depresses ErbB receptor signaling in endothelial cells and cardiac progenitor cells via the promotion of ADAM10-dependent cleavage of ErbB2 at the cell surface, thus contributing to vascular dysfunction and adverse remodeling seen in diabetic patients.

Introduction

Diabetes induces functional changes in the vascular endothelium that result in the development of vascular dysfunction [1]. Hyperglycemia, a hallmark of diabetes, is involved in the induction of oxidative stress, promotion of endothelial cell apoptosis, and increased vascular permeability [2, 3]. Endothelial

dysfunction and low-grade inflammation are associated with increased cardiovascular mortality in diabetes [4]. Patients with diabetes have higher long-term mortality rates after open-heart surgery [5]. We and others recently demonstrated that a higher level of HbA1c, a marker of chronic hyperglycemia, is associated with poor long-term survival in diabetic patients undergoing coronary artery bypass grafting (CABG) [6]. A better understanding of the hyperglycemia-induced pathological alterations in human subjects is necessary to develop novel therapeutic approaches to protect against long-term diabetes-related health problems after cardiovascular surgery.

Animal studies demonstrated a beneficial effect of neuregulins, membrane-bound growth factors belonging to the epidermal growth factor family, in the prevention of cardiovascular complications in diabetes [7]. Neuregulin-1 promotes reparative angiogenesis after ischemic heart injury in experimental diabetes [8]. The level of circulating neuregulin-4 is decreased in patients with diabetes [9], and inversely correlates with HbA1c and diabetic microvascular complications [10, 11]. Neuregulins signal through ErbB receptors. There are four ErbB receptor family members, including ErbB1 (also known as epidermal growth factor receptor or EGFR), ErbB2, ErbB3, and ErbB4 [12]. The expression of all four ErbB receptors has been shown on endothelial cells of various origins [13–16]. Neuregulin-dependent stimulation of ErbB receptors plays an essential role in endothelial cell differentiation [17], survival, proliferation [13], and maintenance of barrier properties [18]. However, the expression of ErbB receptors on cardiac endothelial cells in patients with diabetes and the impact of hyperglycemia on the regulation of ErbB receptors have not been investigated yet.

The goals of this study were to examine the level of ErbB receptors expression on endothelial cells obtained from left ventricular epicardial biopsies of diabetic and non-diabetic patients undergoing CABG surgery and to determine associations between diabetic status, HbA1c level, and ErbB receptor expression. In addition, we evaluated the expression of ErbB receptors on left ventricular epicardial $CD105^{+}$ cells, the subpopulation of cells that may give rise to endothelial cell lineage [17]. Importantly, in addition to ErbB receptors at the cell surface, the total cellular pool also includes newly synthesized and internalized receptors that are not involved in the neuregulin-dependent signaling. To better characterize the effect of diabetes and hyperglycemia, we employed flow cytometric analysis of freshly isolated cardiac cells to specifically determine the level of ErbB receptors cell-surface expression.

Methods

Study subject enrollment

Research was performed in accordance with study protocols approved by Maine Medical Center Institutional Review Board, which is accredited by the Association for the Accreditation of Human Research Protection Programs (AAHRPP). The study cohort consisted of 23 subjects recruited to undergo intra-operative myocardial biopsy at the time of scheduled coronary artery bypass grafting surgery at Maine Medical Center (MMC) in Portland, Maine. All subjects provided informed consent approved by the

MMC Institutional Review Board. All subjects were over 18 years of age. Subjects with known active myocarditis, hypertrophic cardiomyopathy, constrictive pericarditis, significant valvular and/or pericardial disease, severe pulmonary hypertension, significant hepatic disease or renal impairment (creatinine > 2.5 mg/dL), severe ventricular arrhythmias, malignancy other than non-melanoma skin cancers, expected survival less than one year and inability to provide informed consent were excluded. Of the 25 patients that were enrolled in this study, two were excluded for missing LV epicardial biopsies, leaving 23 patients for the data analysis.

Reagents

EBM-2 Basal Medium and EGM-2 SingleQuot Kit supplement/growth factors were purchased from Lonza Walkersville, Inc. (Walkersville, MD), and EGM Cell Growth Medium-2 was prepared according to the manufacturer's instructions. NRG-1 (ECD, 377-HB) and EGF (236-EG) were purchased from Bio-Techne/R&D Systems. TAK-165 and GI 254023X were obtained from Tocris Bioscience (Bristol, UK) and diluted in dimethyl sulfoxide (Cell Culture grade, Sigma). Collagenase II (345 units per mg, CLS-2) was purchased from Worthington Biochemical Corporation (Lakewood, NJ), dispase II (04942078001) was from Roche Life Science (Indianapolis, IN). D-glucose and DNase I was from Sigma (St. Louis, MO). For experiments involving cell stimulation, final concentrations of dimethyl sulfoxide did not exceed 0.1%.

Human microvascular endothelial cells (HMEC-1)

HMEC-1 cells were cultured as previously described [19, 20]. In brief, cells were maintained in M-199 medium supplemented with 15% (v/v) fetal bovine serum and 0.3 µg/ml endothelial cell growth supplement (ECGS, Sigma) under a humidified atmosphere of air-CO_2 (19:1) at 37°C.

For high glucose experiments, HMEC-1 cells were transferred into a 15% FBS M-199 medium without ECGS containing either 5 mM or 25 mM D-glucose.

Blood sample collection

Blood samples from subjects were obtained immediately before surgery, post-anesthesia induction, but prior to skin incision. Venous blood (10 ml) was collected using BD Vacutainer ACD tubes. Small aliquots (50 µl) were used for flow cytometry analysis. Platelet-free blood plasma was prepared at room temperature using two-step centrifugation, each at 2,000 x g for 20 minutes.

LV epicardial biopsy

All procedures for tissue procurement were performed in compliance with institutional guidelines for human research and an institutional review board-approved protocol at MMC. Anterior LV free wall epicardial biopsies (average weight 26.4±1.8 mg) were obtained during planned coronary artery bypass surgery soon after the patient was placed on cardiopulmonary bypass, as described [21, 22]. The epicardial biopsy was placed in a serum-free DMEM medium and kept on wet ice. All samples were processed within 3 hours of collection. All patients were followed postoperatively until discharge. No adverse effects

or post-operative complications ascribable to the biopsy were detected, and all patients were discharged alive.

Preparation of cell suspension from LV epicardial biopsy

Preparation of cell suspension was performed according to a protocol published previously [23]. In brief, minced tissue was incubated in digestion solution (10 mg/ml collagenase II, 2.5 U/ml dispase II, 1 μg/ml DNase I, and 2.5 mM $CaCl_2$) for 45 minutes at 37°C. After passing through a 70-μm cell strainer, the resulting myocyte-free single-cell suspension was centrifuged at 500 x *g*, washed with Dulbecco's PBS, and resuspended in PBS/0.5%BSA/2mM EDTA. Cells were counted, and the cell suspension was divided into two parts. One part was immediately used for flow cytometric analysis, and the other part was used for cell isolation.

Isolation of $CD105^+CD31^-CD45^-$ cells

Endothelial cells and immune cells were labeled with APC-conjugated CD31 (WM59) and CD45 (HI30) and removed from epicardial cell suspension using anti-APC MicroBeads and MS columns (Miltenyi Biotec, Inc.). The $CD105^+$ cells were magnetically isolated from CD31, and CD45 depleted cell suspension using PE-conjugated CD105 (43A3) and anti-PE MicroBeads (Miltenyi Biotec, Inc.). Cells were grown in M199-EGM-2 (3:1, v/v) supplemented with SingleQuotsTM Supplements (CC-4176, Lonza), 10% FBS and 1× antibiotic-antimycotic solution. Cells between passages 2-5 were used for experiments.

For the experiments with high glucose, epicardial $CD105^+CD31^-CD45^-$ cells were transferred into a 10% FBS M199-EBM-2 medium without growth factor supplement, containing either 5 mM or 25 mM D-glucose.

Flow cytometric analysis

Cells (10^6 or less/ml) were treated with Human TruStain FcX™ (Biolegend, San Diego, CA) to prevent non-specific binding, followed by incubation with relevant antibodies for 25 minutes at 4˚C. Cell-surface antigen expression was examined using the following antibodies: CD31 (WM59), CD45 (HI30), and CD105 (43A3) (all from BioLegend, San Diego, CA).

ErbB receptors were assayed using PE-conjugated anti-human ErbB2 (Fab1129P) and IgG2b isotype-matched control (IC0041P), ErbB3 (Fab3481P) and IgG1 control (IC002P), ErbB4 (Fab11311P) and IgG2a (IC003P) isotype control. All anti-ErbB antibodies and controls were purchased from R&D Systems (Minneapolis, MN). All antibodies were titrated to establish high separation between positive and negative cell populations while maintaining a low level of background. Isotype-matched control antibodies were used to determine the level of undesired non-specific binding.

For intracellular staining, cells were fixed and permeabilized using DB Cytofix/Cytopermä (554714, BD Biosciences). The total level of ErbB2 protein was determined using mouse monoclonal Anti-

Neu/ErbB2/HER2 Antibody (3B5) (sc-33684, Santa Cruz Biotechnology) in combination with anti-mouse PE-conjugated IgG (715-116-150, Jackson ImmunoResearch Labs). The level of phosphorylated ErbB2 was measured using rabbit anti-phospho-HER2/ErbB2 (clone: D66B7, Cell Signaling Technology) in combination with anti-rabbit APC-conjugated IgG (711-136-152, Jackson ImmunoResearch Labs).

Fluorescence spillover in multiparametric multicolor flow cytometric analysis was corrected after including all relevant antibodies in the other channels along with an isotype control antibody in a single channel of interest. The expression of cell markers and ErbB receptors was determined after subtraction of the mean fluorescence intensity (MFI) of isotype-matched controls from the MFI of specific antibodies and represented as a delta mean fluorescence intensity (DMFI) as described elsewhere.

Data acquisition was performed on a MacsQuant Analyzer 10 (Miltenyi Biotec., Inc.), and the data were analyzed using WinList 5.0 software. Erythrocytes were lysed with ammonium chloride. Viable and non-viable cells were distinguished using DAPI (to detect dead nucleated cells) and LIVE/DEAD® Fixable Violet Stain kit for detection of non-nucleated cell debris (Life Technologies, Carlsbad, CA).

Analysis of circulating IL-6, IL-8 and TNFa.

The levels of IL-6, IL-8 and TNFa in platelets free blood plasma were measured using Human DuoSet ELISA kits (Bio-techne/R&D Systems).

Statistical analysis

Shapiro-Wilk test was used to determine variable distribution. Comparisons between two groups were performed using two-tailed unpaired *t*-tests (normal distribution) or Mann-Whitney for skewed distribution. Comparisons between three or more groups were performed using one-way or two-way ANOVA with Tukey's multiple comparisons test. For continuous variables, correlation analysis was performed using either Pearson correlation (normal) or Spearman's rank test (skewed distribution correlation). A P-value < 0.05 was considered significant.

Results

Patients

All twenty-three patients underwent cardiac operations with CPB as planned. There were no mortalities. The mean value of age was 62 years with a standard deviation of 13 years. Seventy-four percent of patients were males. There were fifteen patients (65%) with a diagnosis of diabetes mellitus (DM).

Left ventricular epicardial endothelial cells and $CD105^{+}CD31^{-}$ cells express a similar level of ErbB receptors.

The characterization of ErbB receptor expression at the surface of endothelial cells and $CD105^{+}CD31^{-}$ cells was performed in a population of viable single cells obtained from epicardial biopsies, as shown in

Figure 1A. Human immune cells, including neutrophils and monocytes, express a high level of CD31. Some subpopulation of immune cells also coexpresses CD105. The pan-immune marker CD45 was used to distinguish between immune and non-immune cells and avoid the potential misidentification of endothelial cells. The subpopulation of epicardial endothelial cells was defined as $CD31^{+}CD105^{+}CD45^{-}$ cells. Cells that expressed CD105 but not an endothelial marker, CD31, were defined as $CD105^{+}CD31^{-}$ cells within the population of $CD45^{-}$ cells. Non-immune ($CD45^{-}$) cells that did not express CD31 or CD105 were defined as other and were most likely represented by subsets of cardiac fibroblasts and smooth muscle cells. The average percentage of each subpopulation of cells obtained from cardiac biopsies is shown in **Figure 1B**.

Our analysis revealed that all four subtypes of ErbB receptors - ErbB1/EGFR, ErbB2, ErbB3, and ErbB4 - are expressed on both endothelial cells and $CD105^{+}CD31^{-}$ cells (**Figure 1C**). No statistically significant differences were found in the expression of ErbB receptors between epicardial endothelial cells and $CD105^{+}CD31^{-}$ cells.

The cell surface expression of ErbB2 is reduced in patients with DM and inversely correlates with hemoglobin A1C.

To determine potential relationships between the expression of ErbB receptors on endothelial cells and $CD105^{+}CD31^{-}$ cells and the effect of demographic variables and inflammatory markers, we performed a correlation analysis (**Figure 2A**).

Multiple positive associations were found between the expression of ErbB receptors. The levels of both ErbB1 and ErbB4 on endothelial cells were positively correlated to ErbB1, and ErbB4 expressed on $CD105^{+}CD31^{-}$ cells. While not statistically significant, a trend toward positive correlation was found for ErbB2 expression between the two cell types. Positive correlations were also found between the levels of ErbB2, ErbB3, and ErbB4 at the surface of both endothelial cells and $CD105^{+}CD31^{-}$ cells.

The level of ErbB1 and ErbB2 expression on endothelial cells was significantly decreased in patients with DM compared to non-diabetic (non-DM) patients (**Figures 2B and C**). In addition, an inverse correlation was found between the endothelial expression of both ErbB1 and ErbB2 and the level of glycated hemoglobin (**Figures 2D and E**). Also, ErbB2 but not ErbB1 expression on CD150+CD31 cell was inversely associated with HbA1C (**Figure 2F**).

Positive correlations were identified between the expression of ErbB1 on endothelial cells and ErbB2 on CD105+CD31- cells and circulating TNFa. However, the level of TNFa in the blood plasma was low, with a median value of 28 pg/ml and interquartile range from 9 to 89 pg/ml.

High glucose reduces the level of ErbB2 expression at the cell surface of human microvascular endothelial cells and epicardial $CD105^{+}$ cells.

While a pure subpopulation of human LV epicardial cells could be isolated from epicardial biopsies, the majority of $CD31^{+}CD45^{-}$ cells (85% ±5.0%, n=23) undergo apoptosis within 24 hours after the isolation. The rest of the cells can be propagated, but these cells progressively lose endothelial cell markers. For that reason, we used a human microvascular endothelial cell line to determine the effect of high glucose on ErbB receptor expression in endothelial cells. HMEC-1 cells express high levels of ErbB1 and ErbB2 (**Figure 3A**), as do LV epicardial endothelial cells. These levels are down-regulated in patients with DM and elevated HbA1C conditions.

Incubation of HMEC-1 cells in the presence of high glucose for 72 hours resulted in a significantly reduced cell surface expression of ErbB2 but not ErbB1 (**Figures 3B and C**). However, when cells were permeabilized and stained with an antibody against the c-terminus of ErbB2, no differences were found in total ErbB2 expression.

ErbB2 receptors have no ligand binding activity and rely on heterodimerization with other members of the ErbB receptor family to induce intracellular signaling. To determine if the high glucose-induced reduction in the cell surface ErbB2 expression is also associated with decreased activation, we measured phosphorylation of ErbB2 in response to stimulation of cells with EGF, an ErbB1 receptor ligand. EGF induced phosphorylation of ErbB2 in HMEC-1 cells, and this was abrogated in cells co-treated with the specific ErbB2 antagonist, TAK-165 (**Figures 3D and E**). The incubation of HMEC-1 cells with high glucose resulted in a significant decrease in the EGF-induced phosphorylation of ErbB2, consistent with the previously observed reduction in ErbB2 expression on the cell membrane (**Figure 3F)**.

In contrast to endothelial cells, the level of apoptotic cells in the subpopulation of freshly isolated epicardial $CD105^{+}CD31^{-}CD45^{-}$ cells is low (4.1% ± 1.8%, n=23). These cells could also be expanded in the culture without significant changes in the expression of CD105 and ErbB receptors [17]. Similar to endothelial cells, incubation of primary epicardial CD105+ cells with high glucose led to decreased cell surface ErbB2 expression (**Figure 3G**). This was also accompanied by the reduced ability of EGF and NRG-1, ErbB3/ ErbB4 ligands, to induce phosphorylation of ErbB2 (**Figures 3H and I**).

ADAM10 mediates the high glucose-induced reduction in cell surface ErbB2 expression.

Both HMEC-1 and epicardial $CD105^{+}$ non-endothelial cells express ADAM10 (**Figure 4A**), a sheddase that mediates cleavage of the ErbB2 receptors [24]. The expression of ADAM10 and ErbB2 on $CD105^{+}$ cells varies significantly between different patients. Correlation analysis revealed no association between ADAM10 and ErbB2 expression at the cell surface of CD105+ cells (**Figure 4B**), indicating either the absence of baseline ADAM10 activity or ErbB2 protection. Consistent with that, GI 254023X, an ADAM10 inhibitor, did not affect cell surface ErbB2 expression (**Figure 4C**). However, GI 254023X did prevent the effect of high glucose on ErbB2 expression in both HMEC-1 and epicardial CD105+ cells.

Discussion

Our study results reveal that the level of ErbB2 receptors at the surface of epicardial endothelial cells and $CD105^+$ cells is reduced in patients with diabetes mellitus and inversely correlates to the level of glycated hemoglobin. We also demonstrated that high glucose conditions promote ADAM10-dependent shedding and inhibition of ErbB2.

Increasing evidence indicates that ErbB receptors are expressed in different types of cardiac non-myocyte cells in the human heart and play a crucial role in the regulation of heart adaptation to cardiovascular stresses [7]. We previously demonstrated that all four subtypes of ErbB receptors are expressed in cardiac highly proliferative cells [17]. In the current study, we demonstrated that freshly isolated LV epicardial endothelial cells and non-endothelial $CD105^+$ cells are also characterized by the expression of all four receptors - ErbB1, ErbB2, ErbB3, and ErbB4. Non-endothelial $CD105^+$ cells represent a heterogeneous group of cardiac fibroblasts and mesenchymal stem cells and play an important role in angiogenesis [25–28]. Furthermore, we found positive correlations between the ErbB receptors expression on different cell types, most likely reflecting the endothelial origin of cardiac fibroblasts [29] and their generation from common cell types, including cardiac highly proliferative cells [17] and bone marrow progenitors [30]. Considering the role of ErbB receptors in the promotion of cell survival and proliferation [12], a highly similar ErbB receptor signature in both cell types may indicate the necessity of ErbB signaling for coordinated endothelial-stromal cell response to cardiovascular stresses.

Several experimental studies demonstrated that neuregulin-1, acting via ErbB3 and ErbB4, improves glucose homeostasis [31–33]. In the heart, ErbB2 signaling mediates beneficial effects of neuregulin-1 on myocardial angiogenesis and prevention of fibrosis in a rodent model of diabetic cardiomyopathy [8, 34]. It has also been shown that experimental diabetes abrogated up-regulation of ErbB2 in the ischemic heart resulting in worsening of cardiac function[35], indicating the important cardioprotective role of ErbB2 expression in animals. In this study, we demonstrated that diabetes is associated with the down-regulation of ErbB1 and ErbB2 receptor expression on human cardiac non-myocyte cells. Furthermore, we found an inverse association between the level of glycated hemoglobin and ErbB receptors, indicating the potential effect of hyperglycemia in the regulation of cell surface ErbB receptor expression.

The association between ErbB2, hyperglycemia, and diabetes has been previously studied in humans. The level of circulating ErbB2 is associated with hyperglycemia and increased in patients with diabetes[36]. In agreement with this, we found that high glucose decreased ErbB2 expression on cardiac non-myocyte cells through the promotion of ErbB2 cleavage by ADAM10, a principal ErbB2 sheddase [24]. Given the widespread role of ErbB signaling in the maintenance and repair of tissues, it is interesting to consider the potential contribution of widespread ErbB2 shedding for progressive peripheral neuropathy, retinal pathology, and nephropathy associated with poorly controlled diabetes. We have previously demonstrated that saturated fat can dysregulate neuregulin/ErbB signaling in cardiac myocytes via uncoupling of receptor phosphorylation with the PI3K/Akt pathway [37, 38]. Thus there are potentially two mechanisms by which diabetes creates a state of resistance to ErbB signaling.

Emerging evidence indicates that the ErbB1 pathway is involved in diabetes-induced kidney damage and vascular dysfunction [39]. While high glucose conditions did not affect ErbB1 expression at the cell surface in our *in vitro* studies, we cannot exclude the possibility that high glucose regulates the ErbB1 level *in vivo* via as yet unidentified mechanisms. However, our data showed that these mechanisms are not dependent on ADAM10 activity and are different from the effect of high glucose on ErbB2. This is an important finding which may result in the development of therapeutic approaches for specific targeting of different ErbB receptor subtypes. Inhibition of ADAM10 may be beneficial in the prevention of cardiovascular complications of diabetes mellitus via specific protection of ErbB2 expression. In fact, it has recently been shown that inhibition of ADAM10 promotes survival and cardiac function after ischemic injury in mice [40].

ErbB receptors play a crucial role in cardiac cell survival and proliferation [7]. We found that the biopsies from patients with diabetes were characterized by a decreased total number of cells per milligram of tissue, which may indicate increased accumulation of extracellular matrix and fibrotic remodeling [41]. In addition, the number of $CD105^+$ cells was significantly decreased in diabetic patients. We and others previously demonstrated that $CD105^+$ highly proliferative cells could differentiate toward endothelial cell lineage [17, 42]. Reduced populations of $CD105^+$ cells may contribute to impaired regeneration in diabetes.

This study has several limitations. The sample size was small. Therefore, important characteristics of variability among individuals may either be lost or exaggerated. Small sample size can also cause difficulties in finding correlations and demonstrating causation. Nonetheless, this study is among the first to demonstrate the reduced cell surface expression of ErbB receptors on cardiac endothelial cells in patients with diabetes mellitus. Identification of inverse association between ErbB2 and glycated hemoglobin in a small cohort with corresponding complexity and variability makes the strength of our findings even more relevant and clinically important.

Conclusions

In summary, diabetes and hyperglycemia are associated with decreased cell surface expression of ErbB2 on LV epicardial endothelial cells and non-endothelial $CD105^+$ cells. High glucose promotes ADAM10-dependent shedding of ErbB2 from the surface of endothelial cells and non-endothelial $CD105^+$ cells. We suggest that hyperglycemia-driven loss of ErbB2 intracellular signaling results in reduced survival and regeneration of endothelial cells. Our data identified ADAM10 as a new potential therapeutic target for the prevention of diabetic microvascular complications.

Abbreviations

ADAM10 - A disintegrin and metalloproteinase domain-containing protein 10

CABG – coronary artery bypass grafting

DM – diabetes mellitus

EC – endothelial cells

ECGS – endothelial cell growth supplement

EDTA - ethylenediaminetetraacetic acid

EGF – epidermal growth factor

HbA1C - glycated hemoglobin

HMEC-1 – human microvascular endothelial cells 1, dermal

LV – left ventricular

NRG-1 – neuregulin 1

Declarations

Ethics approval and consent to participate

Institutional Review Board approval was obtained, ethics approval was obtained at each participating site, and all patients provided informed consent to participate in the study.

Consent for publication

Not applicable.

Availability of data and materials

The data and materials may be available upon reasonable request.

Competing interests

Not applicable.

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Authors' contributions

MPR, SR, and DBS: designed the study and interpreted the data; JTdK, JC, BS, AMK, ST, EC, DBS, SR and MPR: assisted with an enrollment of subjects, collection of samples, lab work, analysis of clinical and lab data; JTdK, DBS, SR and MPR: wrote the manuscript.

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Figures

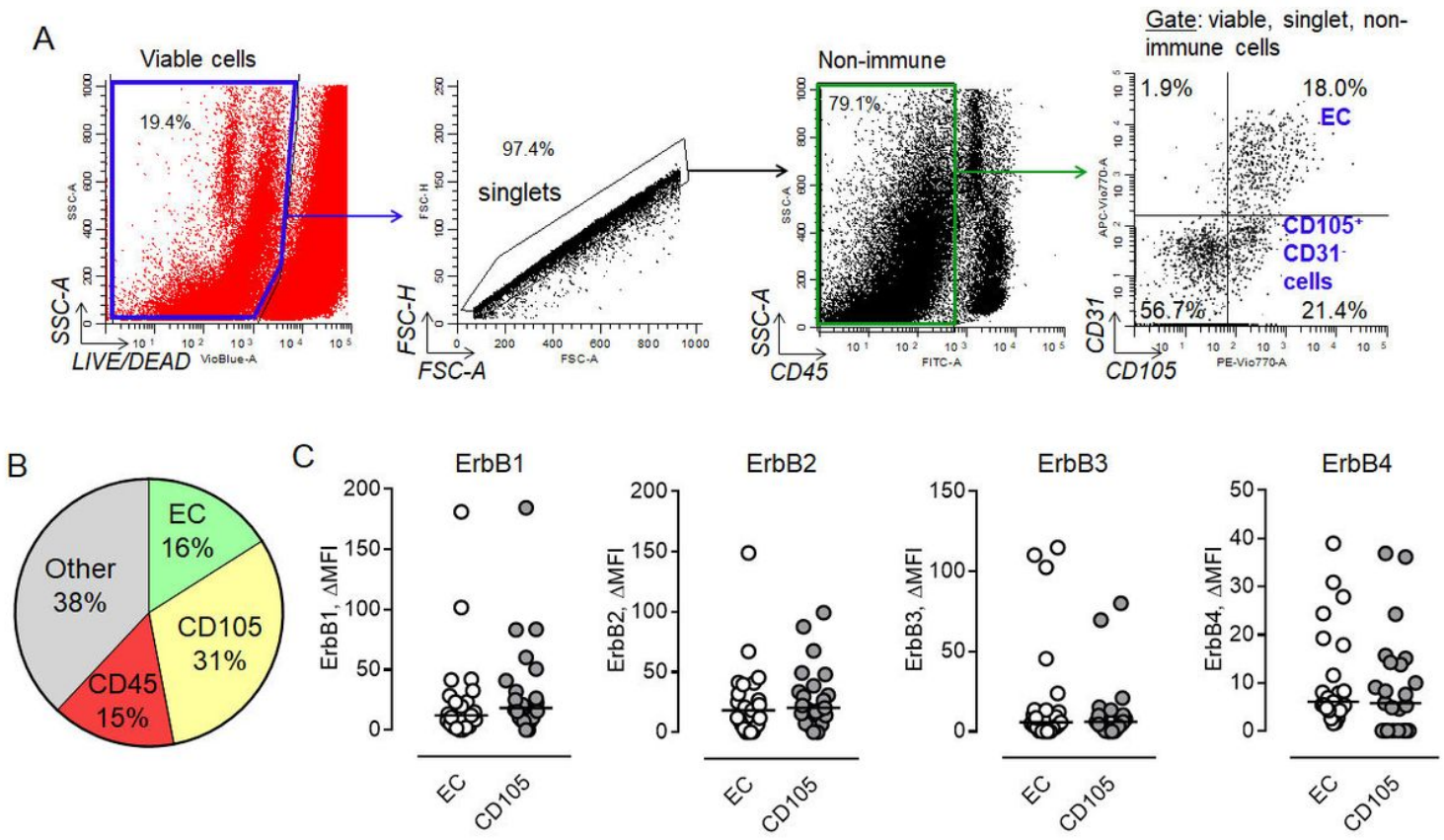

Figure 1

Endothelial cells and CD105+CD31- cells are characterized by a comparable level of ErbB receptors. A. Flow cytometric strategy to determine the expression of ErbB receptors: after enzymatic digestion, viable (left to right: first plot), singlet (second plot), CD45 negative (non-immune, third) cells were analyzed for markers of endothelial cells (CD31, EC) and CD105+CD31- non-endothelial cells (CD105+ cells, fourth plot). The level of ErbB receptors was determined separately in subpopulations of endothelial cells and CD105 cells. B. Graphical representation of mean values of cardiac cell subpopulations obtained from epicardial biopsies. Total number of cells was 4.0 ± 0.5 x 103 cell/mg tissue. The average weight of LV epicardial biopsy was 26 mg. n=23. C. No statistical differences were found in the cell surface expression of ErbB receptors between endothelial cells (EC) and CD105+CD31- cells (CD105). The expression of ErbB receptors was determined after subtraction of the mean fluorescence intensity (MFI) of isotype-matched controls from the MFI of specific antibodies and represented as a delta mean fluorescence intensity (MFI); n=23, Mann-Whitney test.

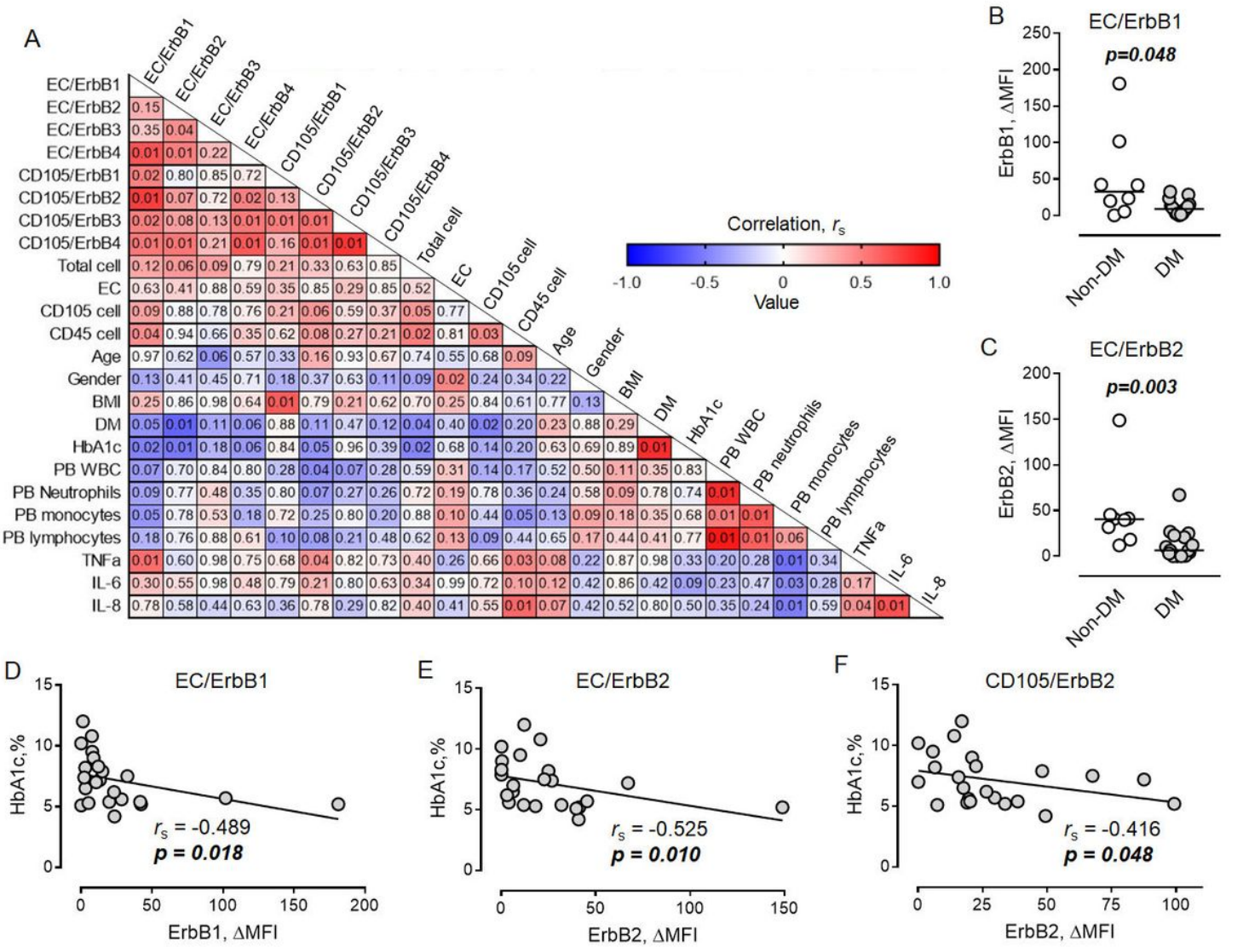

Figure 2

Cell surface expression of ErbB2 is inversely correlated with DM and HbA1c level. A. Heatmap correlation matrix of ErbB receptors expression, subject demographics, and inflammatory markers. Color represents the Spearman correlation coefficient. The value inside each box represents the P-value. B-C. ErbB1 and ErbB2 expression are decreased on endothelial cells in DM (n=15) subjects compared to non-DM (n=8). Mann-Whitney. D-F. Association of ErbB1 (D) and ErbB2 (E-F) cell surface expression on endothelial cells (D-E) and CD105+ cells (F) and HbA1c; n=23, Spearman correlation.

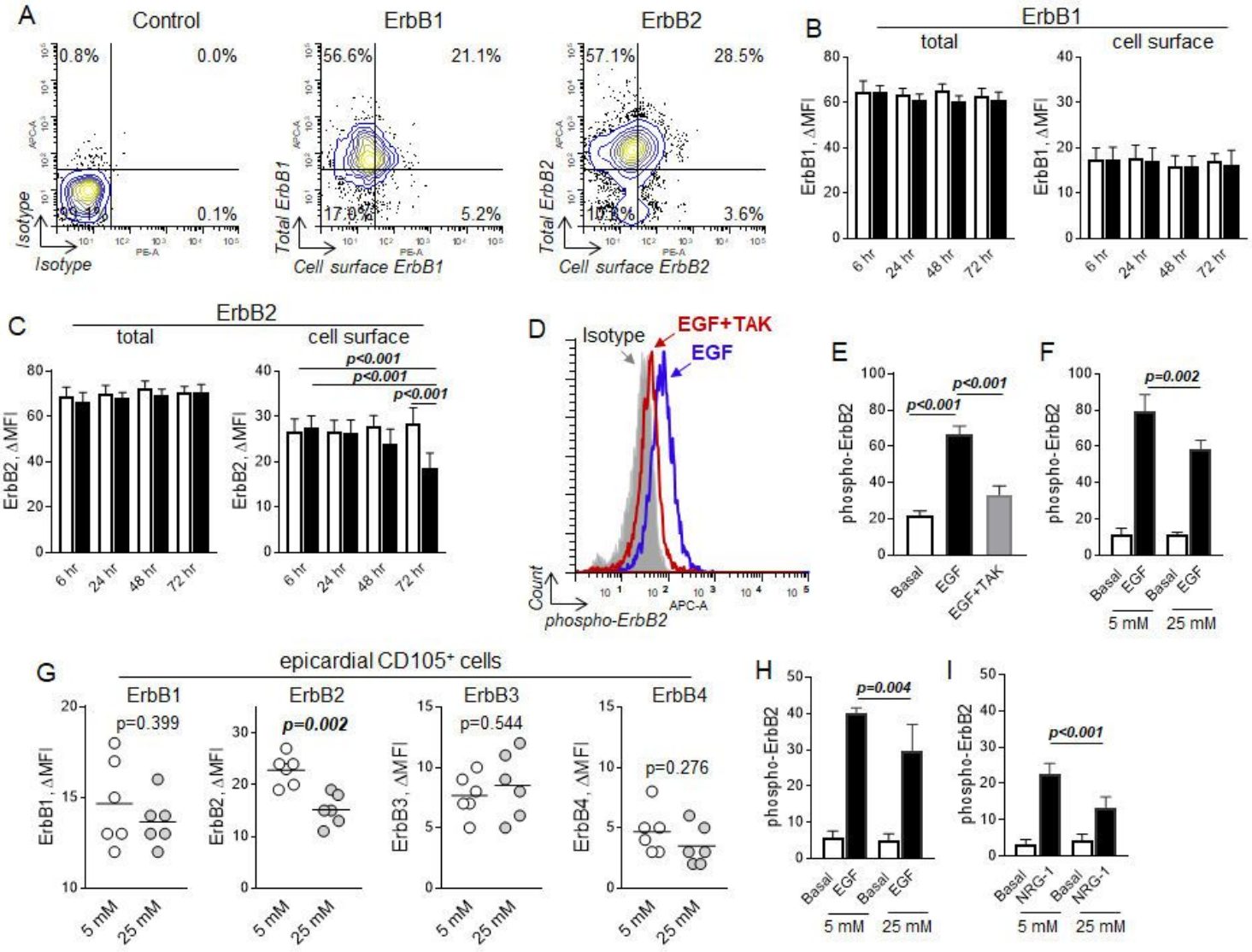

Figure 3

High glucose decreases the ErbB2 expression at the cell surface. A.Representative flow cytometric plots showing the total (after permeabilization) and cell surface (without permeabilization) levels of ErbB1 (middle plot) and ErbB2 (right plot) in HMEC-1 cells. Isotype matched antibodies (left plot) were used to define negative gates. B-C. Graphical representation of total and cell surface levels of ErbB1 (B) and ErbB2 (C) on HMEC-1 cells incubated in the presence of 5 mM (white bars) or 25 mM (black bars) D-glucose for the indicated time; n=9, two-way ANOVA, Tukey's multiple comparisons test. D. Flow cytometric histograms demonstrating the effect of 30 ng/ml EGF alone (blue histogram) and combination of EGF and 300 ng/ml ErbB2 inhibitor, TAK-165 (mubritinib, red histogram) on phosphorylation of ErbB2 in HMEC-1 cells. E.Graphical representation of data from flow cytometric analysis of ErbB2 phosphorylation; n=5, one-way ANOVA. F. HMEC-1 cells were incubated in the presence of 5 mM or 25 mM D-glucose for 72 hrs and stimulated with 30 ng/ml of EGF for an hour. n=5, two-way ANOVA, Tukey's multiple comparisons test. G. Cell surface expression of ErbB receptors on epicardial CD105 highly proliferative cells incubated in the presence of 5 mM or 25 mM D-glucose for 72 hrs, n=6,

Unpaired t test. H-I. Effect of ErbB receptors ligands, 30 ng/ml EGF (H) and 30 ng/ml NRG-1 (I) on ErbB2 phosphorylation in epicardial CD105 highly proliferative cells; n=5, one-way ANOVA, Tukey's multiple comparisons test.

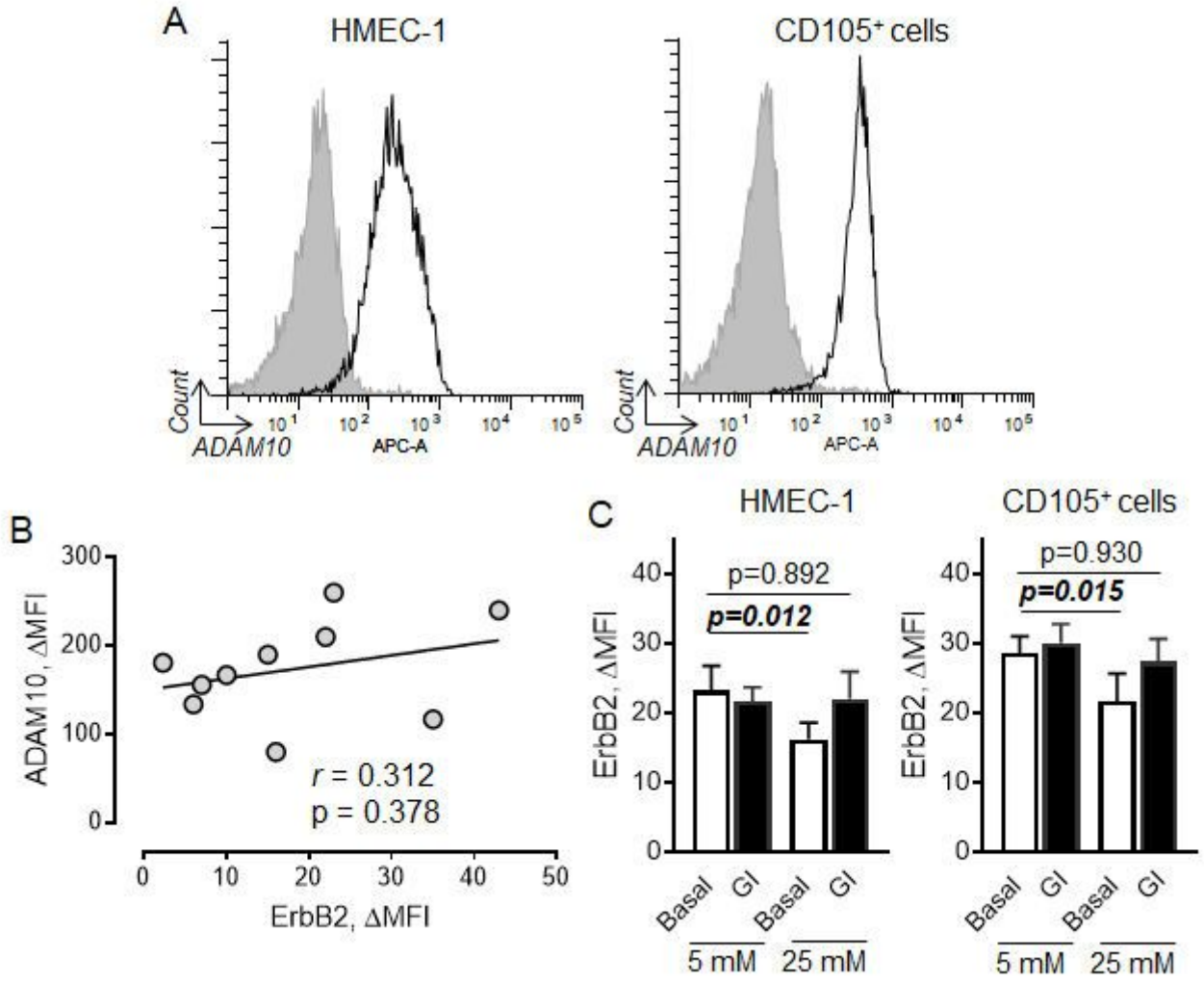

Figure 4

Inhibition of ADAM10 prevents the high glucose-dependent decrease in cell surface ErbB2 expression. A. Expression of ADAM10 on HMEC-1 and CD105+ cells. B. Relationship between the cell surface expression of ADAM10 and ErbB2 in CD105+ cells from CABG patients; n=10. Pearson correlation analysis. C. GI 254023X (3 μM), an ADAM10 inhibitor, rescues ErbB2 expression at the cell surface of HMEC-1 and LV epicardial CD105+ cells incubated in the presence of high glucose; n=5, two-way ANOVA, Tukey's multiple comparisons test.