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Original article

ErbB2 promotes endothelial phenotype of human left ventricular epicardial highly proliferative cells (eHiPC)

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ABSTRACT

The adult human heart contains a subpopulation of highly proliferative cells. The role of ErbB receptors in these cells has not been studied. From human left ventricular (LV) epicardial biopsies, we isolated highly proliferative cells (eHiPC) to characterize the cell surface expression and function of ErbB receptors in the regulation of cell proliferation and phenotype. We found that human LV eHiPC express all four ErbB receptor subtypes. However, the expression of ErbB receptors varied widely among eHiPC isolated from different subjects. eHiPC with higher cell surface expression of ErbB2 reproduced the phenotype of endothelial cells and were characterized by endothelial cell-like functional properties. We also found that EGF/ErbB1 induces VEGFR2 expression, while ligands for both ErbB1 and ErbB3/4 induce expression of Tie2. The number of $CD31^{pos}CD45^{neg}$ endothelial cells is higher in LV biopsies from subjects with high ErbB2 ($ErbB2^{high}$) eHiPC compared to low ErbB2 ($ErbB2^{low}$) eHiPC. These findings have important implications for potential strategies to increase the efficacy of cell-based revascularization of the injured heart, through promotion of an endothelial phenotype in cardiac highly proliferative cells.

1. Introduction

Myocardial response to injury induces a proliferative response that is associated with intense angiogenesis [13,51,62] and cardiac repair, as well as adverse remodeling and development of heart failure [12,26]. Different subpopulations of cardiac cells, including fibroblasts [64], endothelial cells (EC) [62] and cardiac tissue progenitors respond to injury with increased proliferation [60]. Many studies have demonstrated that stimulation of EC proliferation is associated with improved outcomes after experimental cardiac injury [21,27,45,55,58]. In contrast, increased proliferation of fibroblasts may induce a pathological fibrotic process [33,43]. Therefore, better understanding of molecular mechanisms involved in the regulation of cardiac cell type-specific proliferation is required for development of new therapeutic approaches to cure heart disease.

ErbB receptor tyrosine kinase signaling is critical for adult heart function [42] and repair after cardiac injury [4,20]. There are four ErbB receptor family members, including ErbB1 (also known as epidermal growth factor receptor or EGFR), ErbB2, ErbB3 and ErbB4. The ErbB2 is expressed in multiple cardiac cell types. ErbB2-mediated intracellular signaling is reliant upon heterodimerization with ErbB1, ErbB3 or ErbB4 as there is no natural ErbB2 ligand. Both ErbB2 and ErbB4 are found in cardiomyocytes. Neuregulin-dependent activation of ErbB2/4 signaling in cardiomyocytes results in protection against anthracycline- and ischemia-induced injuries [2,14,17]. ErbB2, ErbB3, and ErbB4 are expressed in endothelial cells and regulate angiogenic activity [19,53]. Several studies have demonstrated expression of all four ErbB receptors in cardiac fibroblasts, where they regulate proliferation and paracrine signaling [5,24,31].

We have previously shown that human ventricular myocardium contains a small population of cells which possess remarkable proliferative potential and could be identified by colony-forming ability [54]. Here we examined whether these cells can be isolated from epicardial biopsies and whether they express functional ErbB receptors. We found that human eHiPC express ErbB1-4 receptors that vary among individuals. Experimental studies characterizing baseline and ligand-activated proliferation and phenotype support the hypothesis that ErbB ligands and receptors function in eHiPC to regulate endothelial cell proliferation and phenotype.

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Table 1
Baseline clinical characteristics of study population.

	All subjects (n = 15)
Age (years)	63.6 ± 3.3[a]
Female	13%
Male	87%
Body Mass Index	29.6 ± 1.3
A1C (%)	6.4 ± 0.4
Insulin	7%
Non-insulin antidiabetic agent	73%
ACE-inhibitor or ARB	47%
Beta-blocker	67%
Aspirin	27%
Statin	67%
LVEF < 50	N = 2
LVEF at least 50%	N = 13

[a] Data presented as mean ± SEM, unless otherwise indicated.

2. Methods

2.1. Subjects demographics

The study cohort consisted of 15 patients recruited to undergo intraoperative 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. Demographic data are presented in Table 1.

2.2. 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 the manufacturer's instructions. NRG-1 (ECD, 377-HB) and EGF (236-EG) were purchased from Bio-Techne/R&D Systems. The recombinant human glial growth factor 2 (GGF2; neuregulin-1beta3; USAN - cimaglermin alfa) was provided by Acorda Therapeutics (Ardsley, NY). Calcein AM and 7-AAD were from ThermoFisher Scientific/Molecular probes. AG-1478 and TAK-165 were obtained from Tocris Bioscience (Bristol, UK). AST-1306 was purchased from SelleckChem (Houston, TX). Collagenase II (345 units/mg, CLS-2) was purchased from Worthington biochemical Corporation (Lakewood, NJ), dispase II (04942078001) was from Roche Life Science (Indianapolis, IN) and DNase I was from Sigma (St. Louis, MO). For experiments involving cell stimulation, final concentrations of dimethyl sulfoxide (Cell Culture grade, Sigma) did not exceed 0.1%.

2.3. 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 [11,65]. The epicardial biopsy was placed in serum-free DMEM medium and kept on wet ice. All samples were processed within 3 h of collection. All patients were followed post-operatively until discharge. No adverse effects or post-operative complications ascribable to the biopsy were detected, and all patients were discharged alive.

2.4. Preparation of cell suspension from LV epicardial biopsy

Isolation of cardiac stromal cell populations was performed according to a protocol published previously [54]. 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 min at 37 °C. After passing through a 70-μm cell strainer, the resulting myocyte-free single-cell suspension was centrifuged at 500 × *g*, washed with Dulbecco's PBS, and resuspended in PBS/0.5%BSA/2 mM EDTA. Cells were counted and the cell suspension was divided into two parts. One part was immediately used for flow cytometry analysis and the other part was used to isolate eHiPC.

2.5. Flow cytometric analysis

Cells (10^6/ml) were treated with Human TruStain FcX™ (Biolegend, San Diego, CA) to prevent non-specific binding followed by incubation with relevant antibodies for 25 min at 4 °C. Cell-surface antigen expression was examined using the following antibodies: FITC-conjugated CD45 (HI30), CD90 (Thy-1) and CD105 (43A3), PeCy7-conjugated CD73 (AD2) and CD309 (VEGFR2, 7D4-6), APC-conjugated CD29 (TS2/16), CD117 (c-Kit, 104D2) and CD202b (Tie2, 33.1(Ab33)), and APC/Cy7-conjugated CD31 (WM59) (all from BioLegend, San Diego, CA). FITC-conjugated CD34 (4H11) and CD49f-PeCy7 (eBioGoH3) were from eBioscience/Thermo Fisher Scientific.

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

Table 2
The effect of different cell densities on the number of eHiPC colonies and CD45 cells.

Number of cells seeded per well[a]	Number of wells	Number of colonies[b]	p value[‡]	Number of wells with both colony and non-colony cell expansion	p value[‡]	Number of colonies with $CD45^{pos}$ cells (% of CD45 cell)[c]
50	400	2.6 ± 0.5	–	0.6 ± 0.2	–	nd
100	200	3.2 ± 1.3	ns	1.0 ± 0.3	ns	nd
500	40	3.6 ± 0.9	ns	1.2 ± 0.4	ns	nd
1000	20	5.8 ± 1.2	p < 0.05	1.8 ± 0.4	p < 0.05	1.4 ± 0.7 (3.1 ± 1.2)
2000	10	8.2 ± 1.7	p < 0.05	8.8 ± 0.6	p < 0.001	4.0 ± 1.0 (5.8 ± 2.3)

Colony data represent mean ± SEM from five independent cell isolations (n = 5); nd – not detected.
[a] Cells were seeded in a 48 well culture dish with 1 cm^2 growth area.
[b] Number of colonies was determined on day 7.
[c] Percent of $CD45^{pos}$ cells was evaluated after colonies reached 100% confluence using flow cytometry.
[‡] p-values were calculated using an unpaired *t*-test in comparison to 50 cells/well; ns – not significant.

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