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Original article

STAT3/Pim-1 signaling pathway plays a crucial role in endothelial differentiation of cardiac resident Sca-1+ cells both *in vitro* and *in vivo*

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ABSTRACT

Cardiac stem cells potentially differentiate into cardiac cells, including cardiomyocytes and endothelial cells (ECs). Previously we demonstrated that STAT3 activation by IL-6 family cytokines, such as leukemia inhibitory factor (LIF), induces the endothelial differentiation of cardiac Sca-1+ cells. In this study, we addressed molecular mechanisms for EC differentiation of Sca-1+ cells. First, DNA array experiments were performed to search for the molecules induced by LIF. Among 134 genes that LIF upregulated by more than 4 fold, we focused on Pim-1 gene transcript, because Pim-1 is associated with the differentiation of some cell lineages. Real time RT-PCR analyses confirmed that LIF stimulation upregulated Pim-1 expression. Adenoviral transfection of dominant negative (dn) STAT3 inhibited LIF-mediated induction of Pim-1, while the overexpression of constitutively active STAT3 upregulated Pim-1 expression, suggesting that STAT3 activation is necessary and sufficient for Pim-1 induction. Moreover, in STAT3-deficient Sca-1+ cells, LIF failed to induce Pim-1 expression and EC differentiation. Importantly, the overexpression of dnPim-1 abrogated the induction of EC markers, indicating Pim kinase activity is indispensable for STAT3-mediated EC differentiation in vitro. Finally, Sca-1+ cells labeled with LacZ were transplanted into post-infarct myocardium and the transdifferentiation was estimated. The overexpression of wild-type STAT3 by adenovirus vector significantly promoted EC differentiation, while STAT3 gene ablation reduced the frequency of differentiating cells in post-infarct myocardium. Furthermore, transplanted Sca-1+ cells overexpressing dnPim-1 showed the reduced frequency of EC differentiation and capillary density. Collectively, Pim-1 kinase is upregulated by STAT3 activation in cardiac Sca-1+ cells and plays a pivotal role in EC differentiation both in vitro and in vivo.

1. Introduction

IL-6 family cytokines play important roles in maintenance of cardiac homeostasis. Leukemia inhibitory factor (LIF) and IL-11, IL-6 family cytokines, mediate cardioprotective signal activation through glycoprotein (gp) 130/signal transducer and activator of transcription (STAT) 3 pathway [1–3]. Cardiomyocyte-specific STAT3-deficient mice show higher sensitivity to cardiac injury [4,5], while cardiac-specific transgenic mice expressing constitutively active STAT3 are resistant to myocardial damage [6]. Moreover, activated STAT3 enhances the expression of vascular endothelial growth factor (VEGF) in cardiomyocytes and promotes vessel formation in the hearts [7,8]. Recently, we have demonstrated that IL-6 family cytokines induce endothelial differentiation of resident cardiac Sca-1+ cells, suggesting that these cytokines contribute to neovascularization not only by stimulating pre-existing ECs through autocrine/paracrine system, but by regulating commitment of tissue resident Sca-1+ stem cells into ECs [9,10].

Several kinds of cardiac stem cells have been identified in the adult hearts [11–13]. These cardiac stem cells potentially differentiate into various cardiac cells including cardiomyocytes, smooth muscle cells and ECs. The transplantation of cardiac stem cells into infracted area of hearts promotes cardiac repair and regeneration accompanied by the increase in capillary density. Interestingly, the expression of IL-6 family cytokines is upregulated in infarct myocardium [10], proposing that these cytokines may play some roles in cardiac Sca-1+ stem cell differentiation.

The oncogenic serine/threonine kinase Pim-1 was originally identified as a common proviral insertion site in T and B cell lymphomas [14]. Pim-1 is induced by various cytokines through

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JAK/STAT pathways [15–17]. Pim-1 shows a wide range of biological activities, including cell proliferation, cytoprotection and differentiation [18]. Recently, the overexpression of Pim-1 has been reported to promote proliferation of cardiac progenitor cells [19,20]; however, it remains to be fully elucidated whether Pim-1 regulates the cell fate of cardiac Sca-1+ stem cells.

In this study, we have addressed the regulatory mechanisms of endothelial differentiation of cardiac Sca-1+ cells. And we show that IL-6 cytokine family induces Pim-1 through gp130/STAT3 pathway in Sca-1+ cells and that Pim-1 is required for the endothelial lineage commitment of Sca-1+ cells. These data indicate that gp130/STAT3/Pim-1 pathway regulates endothelial differentiation of resident cardiac Sca-1+ cells after myocardial injury.

2. Materials and methods

2.1. Preparation of cardiac Sca-1+ cells

Sca-1+ cardiac stem cells were prepared by magnetic cell sorting with about 98% purity, as described previously [9]. Sca-1+ cells were isolated from wild type C57Bl/6 mice (10- to 12-week-old, Japan SLC) and *Stat3*$^{flox/flox}$ mice (10- to 12-week-old) [21].

2.2. RT-PCR analysis

RT-PCR was performed as previously described [22]. The PCR primers used in this study were shown in Supplementary Table 1.

Real time RT-PCR was performed with the use of Applied Biosystems StepOne™ Real-Time PCR System (Applied Biosystems, CA) with the Fast SYBR Green PCR Master Mix (Applied Biosystems, CA).

2.3. GeneChip analysis

Gene expression was analyzed using a GeneChip® system with a Mouse Genome 430 2.0 Array, which was spotted with about 39,000 probe sets (Affymetrix, Santa Clara, CA).

2.4. Construction of adenoviral vectors

Adenoviral vectors expressing dominant-negative form of Pim-1 (ad-dnPim-1), dominant-negative form of STAT3 (ad-dnSTAT3), constitutively-active form of STAT3 (ad-caSTAT3), wild-type STAT3 (ad-wtSTAT3) and β-galactosidase fused with a nuclear localization signal (ad-β-gal) were previously described [8,23,24]. Adenoviral vector expressing Cre recombinase (ad-Cre) was kindly gifted from N. Mochizuki (National Cardiovascular Center Research Institute, Osaka, Japan). Sca-1+ cells were infected with adenoviral vectors at a multiplicity of infection (MOI) of 100 for 24 hours, and then cultured under the indicated conditions.

2.5. Western blot analysis

Sca-1+ cells, infected with adenoviral vectors, were stimulated with LIF or IL-11. Cells were harvested and proteins were analyzed by Western blotting, as described previously [25].

2.6. Myocardial infarction and cell transplantation

Myocardial infarction (MI) was generated by ligation of the left coronary artery according to the previous report [3]. Intramyocardial injections of Sca-1+ cells were performed following LAD ligation with minor modification [26]. Intramyocardial injection of 30 μl PBS containing 1.5×10^5 or 3.0×10^5 cells was delivered into two sites of the infarct border zone. Two weeks after transplantation, the hearts were excised and embedded in Tissue-Tek OTC (Sakura) to prepare the frozen sections.

2.7. X-gal staining and immunohistochemistry

The frozen sections (5-μm thick) were prepared from hearts injected with Sca-1+ cells. LacZ staining was performed by overnight incubation of sections at 37 °C. Endothelial cells were identified by immunohistochemical staining with the use of the Vectastain ABC kit (Vector Laboratories) with anti-CD31 antibody (BD Biosciences) or anti-VE-cadherin antibody (BD Biosciences). Cardiomyocytes and smooth muscle cells were identified by anti-α-actinin antibody (Sigma) and anti-α-smooth muscle actin antibody (Sigma), respectively. Ratio of endothelial cell differentiation was estimated as the percentage of CD31+ or VE-cadherin+ in LacZ+ cells. Capillary density was estimated from the number of CD31+ cells. Cell number was counted by the researcher who was blind to the assay conditions.

2.8. Echocardiographic analysis

Two weeks after transplantation, two-dimensional and motion-mode (M-mode) transthoracic echocardiography was performed using a Vevo 770 High-Resolution Imaging System equipped with a 25-MHz transducer (Visual Sonics, Toronto, Canada). Echocardiographic measurements were taken on M-mode in triplicate from at least four separate mice per group. The investigator was blinded to the identity of the mice for analysis.

2.8.1. Statistical analysis

Statistical analyses were performed by Student's t-test or one-way ANOVA with the Bonferroni test. Data were presented at mean ± S.E. or mean ± S.D. pValue <0.05 was considered to be statistically significant.

3. Results

3.1. LIF upregulates Pim-1 expression in cardiac Sca-1+ cells

To explore the downstream targets of gp130/STAT3 in EC differentiation of cardiac Sca-1+ cells, we performed an Affymetrix gene analysis. Since gp130/STAT3 signaling up-regulates the target gene expression within a few hours after IL-6 family cytokine stimulation [27], we analyzed the gene expression 1 hour after LIF stimulation (Supplementary Table 2). From the results of the Affymetrix analysis, we focused on Pim-1 kinase, because Pim-1 kinase is known to be responsible for cell differentiation in several cell lineages [18]. RT-PCR analysis confirmed that Pim-1 expression was significantly upregulated 1 hour after LIF stimulation (Fig. 1A). Pim-1 and Pim-3, but not Pim-2, were rapidly induced by LIF and the expression of these gene transcripts was returned to baseline within 3 hours after LIF stimulation (Fig. 1B). We found that continuous stimulation with LIF for 24 and 48 hours resulted in enhanced mRNA level of Pim-1 (Supplementary Fig. 1). Moreover, the expression of Pim-1 and Pim-3 mRNA were enhanced in a dose-dependent manner (Fig. 1C, and Supplementary Fig. 2). These results indicate the expression of Pim-1 and −3 is regulated by LIF in cardiac Sca-1+ cells. Next, we examined whether IL-6 and IL-11 induce Pim family in cardiac Sca-1+ cells. IL-11, but not IL-6, can induce EC differentiation of Sca-1+ stem cells through STAT3 activation, because IL-11 receptor is expressed in Sca-1+ cells while not IL-6 receptor [10]. IL-11 stimulation induced both *Pim-1* and *Pim-3* gene expression, while IL-6 failed to up-regulate those genes (Fig. 1D, and Supplementary Fig. 3). *Pim-2* gene expression was not enhanced by stimulation of IL-11 nor IL-6 (Supplementary Fig. 3). Moreover, we confirmed that Pim-1 protein level was increased by LIF or IL-11 treatment as well (Fig. 1E, F).