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Signals through gp130 upregulate Wnt5a and contribute to cell adhesion in cardiac myocytes

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Abstract Glycoprotein 130 (gp130), a common receptor of IL-6 family cytokines, plays critical roles in cardiac functions. Here, we demonstrate that the stimulation of gp130 with leukemia inhibitory factor (LIF) promoted cell adhesion in a cadherin-dependent manner in cultured cardiomyocytes. Wnt5a was upregulated by the stimulation of gp130 with IL-6 family cytokines, accompanied by N-cadherin protein upregulation. Wnt5a was not induced by LIF in cardiomyocytes expressing dominant-negative STAT3. Ablation of Wnt5a by antisense cDNA inhibited LIF-induced cell adhesion. Collectively, signals through gp130 upregulate Wnt5a through STAT3, promoting the N-cadherin-mediated cell adhesion.

Keywords: IL-6; STAT3; Wnt; N-cadherin; Cytokine; Cardiomyocyte

1. Introduction

Glycoprotein 130 (gp130) is a common receptor of interleukin-6 (IL-6) family cytokines. Signals through gp130 are transduced to signal transducer and activator of transcription (STAT) [1,2]. In cardiac myocytes, gp130 is activated by some kinds of IL-6 related cytokines, such as leukemia inhibitory factor (LIF), cardiotrophin-1 (CT-1), through LIF receptor [3]. Signals through gp130 regulate the transcription of cytoprotective genes, including *bcl-xL* [4], *vascular endothelial growth factor* (VEGF) [5], and *manganese superoxide dismutase* (MnSOD) [6], through STAT pathway. Thus, it is postulated that gp130/STAT pathway prevents cardiac myocytes from myocardial degeneration. Importantly, STAT pathway is activated under pathological stresses, such as hypoxia [7], mechanical stretch [8] and catecholamine stimulation [9].

Cadherin family proteins are the key regulators in cell adhesion. N-cadherin is abundantly expressed in the heart and localized at intercalated disks [10]. In cultured cardiomyocytes, blocking antibody against N-cadherin leads to the disturbance of myofibrillogenesis [11]. Pathological analyses demonstrated that the disruption of the intercalated disks is related with cardiac disorders, such as dilated cardiomyopathy [12–14], consistent with the previous report that mice lacking N-cadherin show the cardiac abnormality [15]. Thus, it could be proposed that the cell adhesion through cadherin system is essential for maintenance of cardiac homeostasis.

Accumulating evidences have demonstrated that Wnt family proteins modulate cadherin function [16]. Interestingly Wnt3 and Wnt5a can regulate N-cadherin function and promote cell adhesion in cardiac myocytes [17]. However, biological significances of Wnt/cadherin system remain to be fully understood in cardiomyocytes because of limited information about regulatory mechanisms of Wnt family expression.

Recently, it was reported that loss of function mutation of STAT pathway in *Drosophila* shows the impaired motility of ovarian epithelial cells with the downregulation of D-cadherin [18]. Here, to address the possibility that gp130/STAT signaling crosstalks with cadherin system, we examined the involvement of gp130/STAT in cell adhesion system in cardiac myocytes. And it has been demonstrated that the signals through gp130 upregulate Wnt5a with the induction of N-cadherin protein and modulate cell–cell interaction.

2. Materials and methods

2.1. Cell culture and reagents

Primary cultures of rat neonatal cardiac myocytes were prepared as described previously [4]. More than 90% of the cultured cells consisted of cardiac myocytes, analyzed by immunofluorescent microscopy (data not shown).

In the experiments concerning the signaling pathways downstream of gp130, wortmannin (Nacalai, Japan) and U0126 (Cell Signaling, MA) were used. Wortmannin is widely used as an inhibitor for PI3 kinase [19]. U0126 specifically inhibits MEKs 1 and 2, resulting in the inhibition of ERKs 1 and 2 (Cell Signaling, MA) [20].

LIF was purchased from Chemicon International (CA). Biological activity of 50 U/ml is defined as the concentration that induces the differentiation of 50% of M1 myeloid leukemic cells, according to the product protocol. CT-1 and IL-6 were from Peprotech EC (London, UK).

2.2. Cell adhesion assay

Cell adhesion was estimated by cell aggregation assay described previously [21] with minor modification. In brief, after stimulated with various kinds of reagents, cells were washed twice with PBS. Single cell

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suspension was prepared by incubating with PBS containing 5 mM EGTA and 5 mM EDTA. After centrifuged at 800 rpm for 5 min, cells were re-suspended in culture medium. Cell suspension (4×10^5 cells/ml) was rotated for 1 h and plated on culture dishes at 4×10^5 cells/cm^2 for 30 min. Cell aggregations were counted in number. The myocyte masses with more than 100 μm diameter were defined to be cell aggregation.

2.3. Adenoviral vectors

Adenoviral vector expressing dominant-negative STAT3 (dnSTAT3) was constructed as described previously [22].

Dominant-negative N-cadherin cDNA, which encodes truncated N-cadherin molecule consisting of a large deletion of the extracellular domain, was gifted by Dr. Jeffrey I. Gordon (Washington University) [23]. Adenovirus vector expressing dominant-negative N-cadherin was generated as described previously [24]. In brief, dominant-negative N-cadherin cDNA was subcloned into pCCMVpLpA vector and the resulting plasmid was co-transfected with pJM17 into 293 cells to allow for homologous recombination. The adenovirus vector was cloned from a single plaque and amplified in 293 cells. The vector was purified by ultracentrifugation in the presence of CsCl.

Adenovirus vector expressing sense or antisense Wnt5a cDNA tagged with HA (Upstate, NY) was generated by inserting Wnt5a cDNA into pCCMVpLpA in sense or antisense direction, followed by the cotransfection with pJM17 into 293 cells, as described above.

Cardiac myocytes were transduced by the adenovirus vector at MOI 20 as described previously [25]. At this MOI, more than 90% of myocyte population was transduced (data not shown).

2.4. Immunoblot analysis

Cell lysates from cultured cardiac myocytes were generated by adding 100 μl of SDS sample solution per 35 mm culture dish. Proteins were separated by SDS–PAGE and transferred onto a polyvinylidene difluoride membrane. After blocking in TBS-T (150 mM NaCl, 50 mM Tris, and 0.1% Tween 20, pH 7.4) containing 2% skimmed milk, membranes were probed with anti-Wnt5a (R&D system, MN), anti-Cdk4, anti-T-cadherin, anti-HA (Santa Cruz Biotechnology, CA), or anti-N-cadherin (BD Science, CA) antibody. ECL system (Amersham, NJ) was used for the detection.

2.5. Northern blot analyses

Northern blot analyses were performed as described previously [5]. The Wnt5a probe was generated by RT-PCR using Reveraace (TOYOBO, Japan). PCR was performed with primers (forward; GGA GGT GCC ATG TCT TCC AAG T, reverse; ATG CTC GGC TCA TGG CGT TCA C). PCR product was subcloned into PCR2.1 vector (Clontech, CA). *Eco*RI fragment of the plasmid was used after its sequence was confirmed. Total RNA was prepared with the acid guanidinium–phenol–chloroform method. Ten micrograms of total RNA was used for the Northern blotting, as described previously [5].

3. Results and discussion

3.1. LIF induces cell aggregation of cultured cardiomyocytes in a cadherin-dependent manner

To address the biological effects of LIF on cell–cell adhesion, we examined the cell adhesion activity of LIF by cell aggregation assay described previously [21]. As shown in Fig. 1, LIF stimulation resulted in cell aggregation in cardiomyocytes transduced with adenovirus vector expressing β-galactosidase (β-gal), a control vector, while adenoviral transduction of dominant-negative N-cadherin inhibited LIF-induced cell aggregation.

3.2. Signals through gp130 upregulate Wnt5a expression accompanied by N-cadherin induction

In cardiac myocytes, cell–cell adhesion through cadherin pathway is regulated by Wnt family proteins [17,26]. In the preliminary assays, we examined the expression of Wnt1, 3, 5a, 8 and 11 in cardiac myocytes by RT-PCR. Wnt1, 3 and 8 mRNAs were not detected in cultured cardiac myocytes.

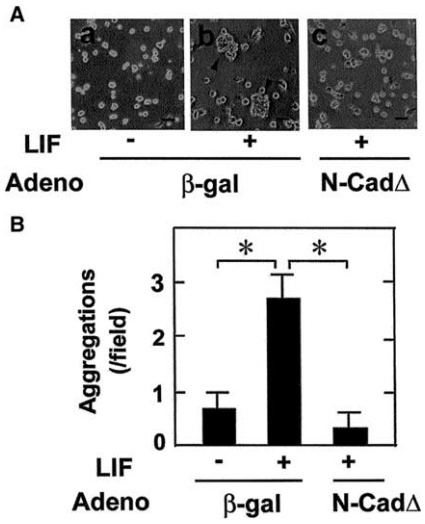


Fig. 1. LIF induces cardimyocyte aggregation in a cadherin-dependent manner. Cardiomyocytes, transduced with adenovirus expressing dominant-negative N-cadherin (N-CadΔ) or β-gal, were stimulated with LIF for 24 h. Single cell suspensions were prepared. Cell aggregation assay was performed as described in Section 2. (A) Representative phase contrast micrographs were shown (magnification ×40, bar 100 μm). Cardiomyocytes transfected with β-gal show low number of cell aggregations in the absence of LIF (a), while LIF induces cell aggregations with more than 100 μm diameter (arrowheads) (b). Note that dominant-negative N-cadherin inhibited the cell aggregation induced by LIF (c). (B) Cardiomyocyte aggregations were counted in seven visual fields. Data were presented as means ± standard error. $^*p < 0.05$.

Wnt11 mRNA was detected but its expression was not influenced by LIF, while Wnt5a was expressed in cardiomyocytes and increased by LIF stimulation (data not shown). Thus, by Northern blotting analyses, we confirmed that Wnt5a mRNA was 2.7-fold upregulated in response to LIF (Fig. 2A(a) and (b)). Importantly, Wnt5a protein was also induced by LIF in parallel to mRNA (Fig. 2B).

As Wnt5a is reported to stabilize N-cadherin protein [17], we examined the effects of LIF on the expression of N-cadherin protein. Cultured cardiac myocytes were stimulated by LIF. Cell lysates were prepared and immunoblotted with anti-N-cadherin antibody. As shown in Fig. 3, N-cadherin protein was upregulated by LIF in a dose-dependent manner, but Cdk4, an internal control, was not affected by LIF. To rule out the possibility that LIF upregulates N-cadherin mRNA, we performed Northern blotting for N-cadherin, however, no induction of N-cadherin mRNA was detected (data not shown). T-cadherin is reported to be expressed in cardiac sarcolemma [27]. However, the bands with 100 and 120 kDa, corresponding to T-cadherin, were not affected by LIF stimulation (data not shown).

3.3. CT-1, but not IL-6, induces Wnt5a and promotes cell aggregation

To make clear whether or not Wnt5a and N-cadherin are induced by other signals through gp130, we examined the effects of IL-6 and CT-1. Cell lysates from cardiomyocytes stimulated with IL-6 and CT-1 were immunoblotted with anti-N-cadherin, anti-Wnt5a and anti-Cdk4 antibodies. CT-1 binds to LIF receptor in cardiomyocytes [3], followed by the remarkable activation of gp130 signaling pathways, while IL-6

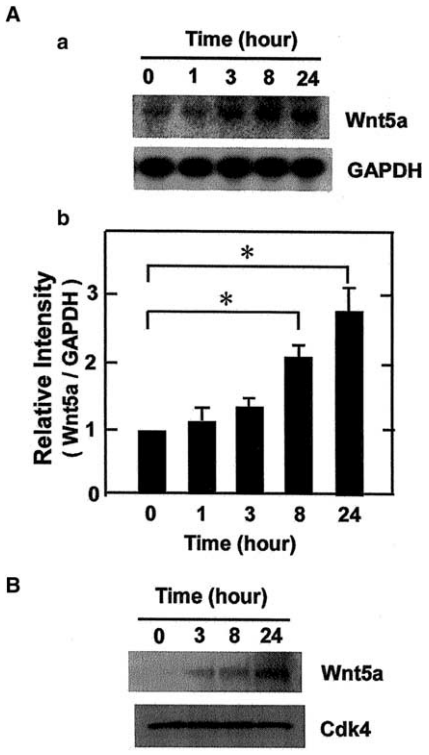


Fig. 2. LIF upregulates Wnt5a mRNA and protein in cultured cardiomyocytes. Cultured cardiomyocytes were incubated with 1000 U/ml LIF for the indicated time. (A) Total RNA was prepared and Northern blotted for Wnt5a and GAPDH. (a) Representative data are shown. (b) Quantitative analyses of Wnt5a mRNA expression. The band intensity of Wnt5a was normalized with that of GAPDH. Ratio of Wnt5a expression to control was calculated. Data were presented as means ± standard error. $^{*}p < 0.05$ ($n = 3$). (B) Cell lysates were immunoblotted with anti-Wnt5a and anti-Cdk4 antibodies. The experiments were repeated three times with similar results.

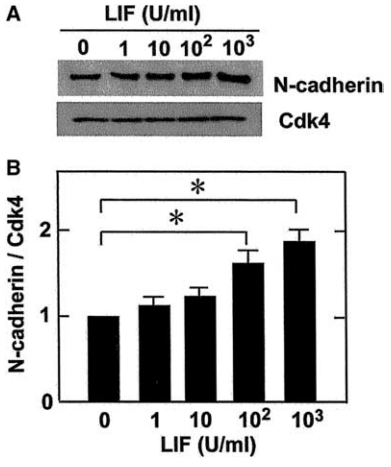


Fig. 3. Stimulation of gp130 by LIF upregulates Wnt5a and N-cadherin in cardiac myocytes. Cultured cardiac myocytes were stimulated with the indicated concentrations of LIF for 24 h. Cell lysates were prepared and immunoblotted with anti-N-cadherin and anti-Cdk4 antibodies. (A) Representative data are shown. (B) Quantitative analyses of N-cadherin expression. The band intensity of N-cadherin was normalized with that of Cdk4. Ratio of N-cadherin expression to that of control was calculated. Data were presented as means ± standard error. $^{*}p < 0.05$ ($n = 3$).

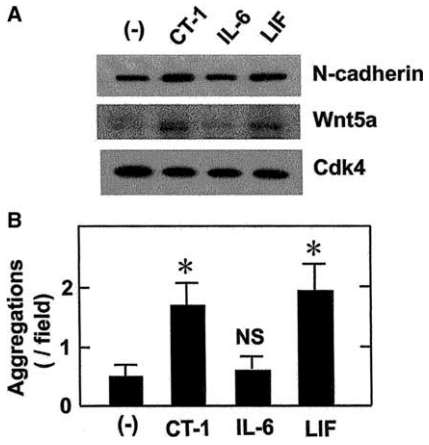


Fig. 4. Cardiotrophin-1, but not inteleukin-6, activates Wnt5a/N-cadherin pathway and promotes cell adhesion. Cardiac myocytes were stimulated with CT-1 (20 ng/ml), IL-6 (20 ng/ml), or LIF (1000 U/ml) for 24 h. (A) Cell lysates were prepared and immunoblotted with anti-N-cadherin, Wnt5a and Cdk4 antibodies. The experiments were repeated three times with similar results. (B) Cells were harvested and cell aggregation assay was performed as described in Section 2. Data were presented as means ± standard error. $^{*}p < 0.05$ versus non-treated cells (–). NS, not significant.

activates gp130 to a lesser extent because of low level expression of IL-6 receptor in cardiac myocytes [5]. In parallel, stimulation of cardiac myocytes with CT-1 upregulated Wnt5a and N-cadherin, but not that of IL-6 (Fig. 4A). Next, we examined the effects of CT-1 or IL-6 on cell aggregation. As shown in Fig. 4B, CT-1 promotes the cell aggregation, but not IL-6, concomitant with the induction of Wnt5a. These findings suggest that gp130-mediated upregulation of Wnt5a is closely associated with the increase in cell aggregation activity.

3.4. STAT3 is required for LIF-mediated induction of Wnt5a

Next, we analyzed the signaling pathways responsible for Wnt5a induction using adenovirus vectors expressing dnSTAT3. Wnt5a mRNA was upregulated by LIF in β-gal-transfected cell, but not in the cardiomyocytes overexpressing dnSTAT3 (Fig. 5A), suggesting that STAT3 activity is re-

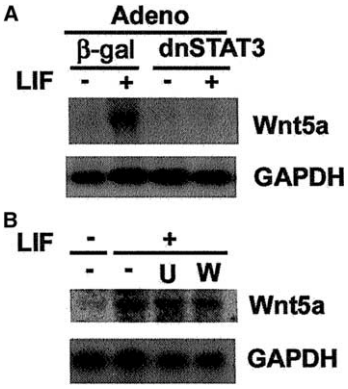


Fig. 5. LIF upregulates Wnt5a expression through STAT3. (A) Cultured cardiomyocytes were transduced with adenovirus vectors expressing dnSTAT3 or β-gal, as a control, at MOI 20 and cultured with (+) or without (−) LIF. Total RNA was prepared and Northern blotted for Wnt5a and GAPDH. (B) Cardiomyocytes, pretreated with U0126 (2 μM) or wortmannin (200 nM) for 30 min, were stimulated with 1000 U/ml LIF for 24 h. Total RNA was prepared and Northern blotted for Wnt5a and for GAPDH.

quired for the Wnt5a induction by LIF. We also examined the influences of ERK and PI3 kinase on Wnt5a mRNA induction in response to LIF by using U0126 and wortmannin, respectively, as described in Section 2. As shown in Fig. 5B, neither inhibition of ERK nor of PI3 kinase affected the Wnt5a induction by LIF.

3.5. LIF-induced upregulation of Wnt5a contributes to cell aggregation

To evaluate the importance of Wnt5a in gp130-mediated cardiomyocyte adhesion, we generated adenovirus vector expressing sense or antisense Wnt5a mRNA. As shown in Fig. 6A, N-cadherin was 2.3-fold upregulated by overexpression of Wnt5a in sense direction, as reported previously [17]. In contrast, adenoviral gene transfer of antisense Wnt5a cDNA abrogates LIF-mediated induction of Wnt5a, accompanied by the downregulation of N-cadherin, but not that of β-gal, a control. Next, we transduced the adenovirus vectors expressing sense and antisense Wnt5a, dnSTAT3, and β-gal into the cardiac myocytes and cell aggregation activity was analyzed (Fig. 6B). The overexpression of sense Wnt5a promoted cell adhesion even in the absence of LIF, as reported [17]. LIF did not promote cell aggregation in cardiomyocytes transfected with antisense Wnt5a cDNA. Moreover, dnSTAT3 also abrogated LIF-induced cell aggregation. These findings support the causality between STAT3-mediated induction of Wnt5a and cell aggregation.

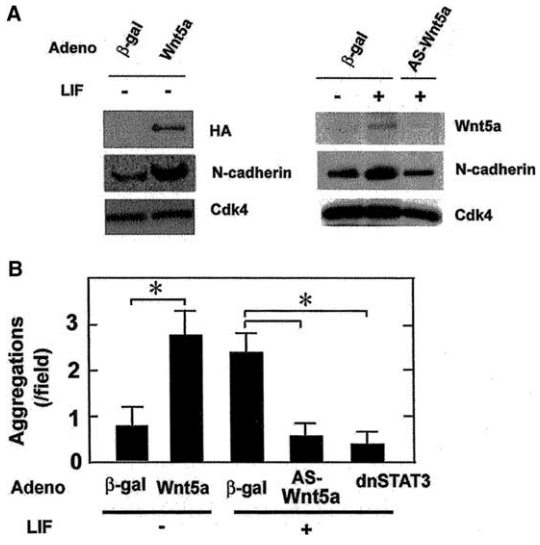
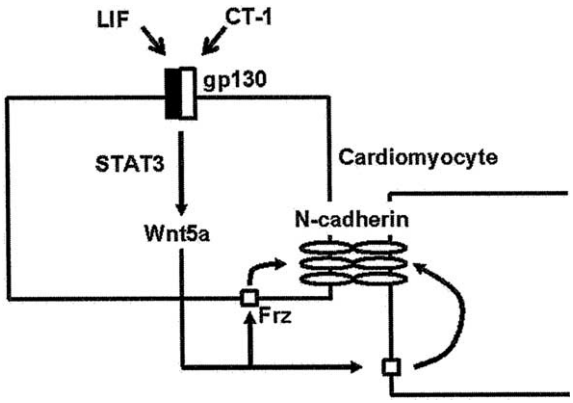


Fig. 6. LIF-mediated induction of Wnt5a is required for cardiomyocyte aggregation. (A) Adenovirus vector expressing sense or antisense Wnt5a cDNA was generated. Cardiac myocytes were transduced with adenovirus vector expressing sense (Wnt5a) or antisense (AS-Wnt5a) Wnt5a cDNA. Adenovirus vector overexpressing β-gal was used as a control. Twenty-four hours after transduction, cells were treated with or without 1000 U/ml LIF for 24 h. Cell lysates were prepared and immunoblotted with anti-N-cadherin, anti-Wnt5a, anti-HA or anti-Cdk4 antibody. (B) Cells were transduced by adenovirus vectors expressing β-gal, Wnt5a, AS-Wnt5a, and dnSTAT3. Twenty-four hours after transduction, cells were treated with or without 1000 U/ml LIF for 24 h. Cells were collected and cell aggregation assay was performed as described in Section 2. Data were presented as means ± standard error. $^{*}p < 0.05$.



Scheme 1. Signals through gp130 regulate N-cadherin pathway through Wnt5a. Activation of gp130 by IL-6 family cytokines, such as LIF and CT-1, induces Wnt5a through STAT3. Wnt5a promotes cell–cell adhesion in an N-cadherin dependent manner. Frz; Frizzled Wnt receptor [17].

3.6. Signals through gp130 regulate Wnt5a/N-cadherin pathway

In the present study, we have demonstrated that signals through gp130 upregulated Wnt5a and promoted cardiomyocyte adhesion via N-cadherin pathway. We also revealed that STAT3 activity is required for Wnt5a induction. Moreover, ablation of Wnt5a inhibited the upregulation of N-cadherin by LIF with the abrogation of gp130-mediated cell adhesion, proposing the functional relation between Wnt5a induction and cell adhesion through N-cadherin in gp130 signaling (Scheme 1).

Target disruption of *gp130* gene results in myocardial abnormality [28], suggesting that gp130 is physiologically involved in cardiac functions. Indeed, it has been demonstrated that activation of gp130 transduces the cytoprotective signals [4–6]. Recently, it has been demonstrated that activation of gp130 by LIF promotes cardiac regeneration after myocardial infarction [29]. Importantly, the formation of adherens junction through N-cadherin is a critical step for the cardiac regeneration from the damage [30]. Collectively, here, we propose a novel role of gp130 signals as a regulator of cadherin systems, which might contribute to the maintenance of cardiac homeostasis, including cardiac repair.

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