日本財団 助成事業
The Nippon Foundation

財団法人日中医学協会
2004年度共同研究等助成金－在留中国人研究者－報告書

2005 年 3 月 3 日

財団法人　日 中 医 学 協 会　御中

貴財団より助成金を受領して行った研究テーマについて報告いたします。

添付資料：**研 究 報 告 書**

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1．**助 成 金 額**：600,000 円

2．**研究テーマ**　コロニー刺激因子（G-CSF）における心筋梗塞の治療効果の検討

3．**成果の概要**（100字程度）

我々は、最近 G-CSF が心筋梗塞後心筋リモテリングや、心機能の低下を抑制することを報告した。本研究では、心臓を構成する細胞の中で最も細胞数が多い線維芽細胞に対する G-CSF の作用を検討した。G-CSF は線維芽細胞内のシグナル伝達を惹起し、線維芽細胞が産生する collagen を減少することにより心臓リモテリングを抑制することが認められた。

4．**研 究 業 績**

(1) 学会における発表　(無)・有（学会名・演題）

(2) 発表した論文　(無)・有（雑誌名・題名）

－日中医学協会助成事業－

コロニー刺激因子（G-CSF）における心筋梗塞の治療効果の検討

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Abstract

Background: Many cytokines have been reported to be increased in human and animal models with cardiovascular diseases. Myocardial infarction (MI) is accompanied with an inflammatory reaction that induces cardiac dysfunction. The inflammatory reaction has been investigated in animal models of MI or myocardial ischemia reperfusion injury. Several hematopoietic growth factors including granulocyte colony-stimulating factor (G-CSF) have been reported to be positive regulators of granulopoiesis and act at different stages of myeloid cell development. Recently, it has been reported that the treatment with G-CSF promotes mobilization of bone marrow stem cells to the injured heart and induces myocardial regeneration after MI. We also examined the beneficial effects of G-CSF after MI in the mice and large animal models. Our data show that G-CSF prevents left ventricular remodeling and dysfunction after MI but no evidence indicting that G-CSF induces cardiac homing of bone marrow cells in the infracted heart. Therefore, it is very important to elucidate whether G-CSF acts directly on cardiomyocytes and cardiac fibroblasts and the mechanism of how G-CSF prevents remodeling after MI.

Methods and results: In the present study, we first detected G-CSF receptor (G-CSFR) by the method of reverse transcriptional-polymerase chain reaction (RT-PCR) or immunofluorescence staining on cardiomyocytes and cardiac fibroblasts. We examined the effect of G-CSF on intracellular signalings in cultured neotatal rat cardiac fibroblasts by using of western blot method. We also examined the protein level of matrix metalloproteinases (MMPs), collagen type I, and smooth muscle (SM) α-actin by western blot analysis. Our data showed that G-CSFR was detected on cultured cardiomyocytes and cardiac fibroblasts both by RT-PCR and immunofluorescence staining. Incubation of cardiac fibroblasts with G-CSF (100ng/ml) significantly downregulated the protein level of MMPs, collagen type I, and SM α-actin. Western blot analysis also revealed that Jak2/Stat3, PKCζ, ERK 1/2, and Akt1 were significantly activated in a time-dependent manner by G-CSF treatment.

Conclusion: G-CSFR also to be expressed on cardiomyocytes and cardiac fibroblasts. G-CSF exerts directly on cardiac fibroblasts through G-CSFR and induces activations of the Jak2/Stat3, PKCζ, ERK 1/2, and Akt1 signaling pathways, resulting in down-regulation of MMPs, collagen, and SM α-actin protein expression in the cultured cardiac fibroblasts.

Key words: G-CSF, cardiac fibroblasts, signaling pathway, MMPs, collagen.

Introduction

Myocardial infarction (MI) is the most common cause of cardiac morbidity and mortality in many countries. Left ventricular remodeling after MI causes progression to heart failure and leads to death ultimately. Therapeutic advances have been mainly targeted at restoring antegrade perfusion in the infarct-related artery, but there seems to be a ceiling of benefit [1]. It has been recently reported that some cytokines including granulocyte colony-stimulating factor (G-CSF), erythropoietin (EPO), and leukemia inhibitory factor (LIF) are beneficial on the cardiac remodeling after MI [2-5]. Accordingly, an alternative approach by cytokines maybe promise therapy of MI.

Combination therapy of stem cell factor (SCF) and G-CSF has been reported to improve cardiac dysfunction and reduces mortality after MI in mice [2]. We also observed G-CSF prevents left ventricular remodeling and dysfunction after MI in the mice and large animal models [6-7]. Therefore, it is of particular importance to determine the molecular mechanism of how G-CSF exerts beneficial effect on the heart. Previous studies show that G-CSFR only to be expressed on blood cells such as myeloid leukemic cells, leukemic cell lines [8]. But it has not been elucidated whether G-CSFR is expressed on cardiomyocytes and cardiac fibroblasts.

The healing process after MI begins from absorption of necrotic tissues, moves into granulation with numerous myofibroblasts, rich microvessels, and collagen, and then forms scar tissues consisting

primarily of collagen, with rare vessels via apoptosis of granulation cells [9]. Several studies demonstrated that G-CSF prevented cardiac remodeling and dysfunction at least in part, by improving early post-infarct ventricular expansion through promotion of reparative collagen synthesis in the infracted area [10-11]. Accordingly, we hypothesized that maybe the degradation of extracellular matrix (ECM) by MMPs contributed to the beneficial effects of G-CSF on MI wounds healing.
In the present study, we demonstrate G-CSF exerts directly on cardiac fibroblasts via G-CSFR that is also expressed on cardiomyocytes and cardiac fibroblasts. G-CSF downregulates the MMPs and type I collage, and smooth muscle (SM) α-actin protein levels and activates Jak2/Stat3, PKCζ, ERK 1/2, and Akt1 signaling pathways.

Methods

Cell Culture

Cardiac fibroblasts were prepared from one-day-old Wistar rats as described [12]. Cardiac fibroblasts were obtained from adherent cells on the preplating dish. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin solution, plated 10 ml onto 100-mm plastic culture dishes and cultured at 37 °C in 95% air / 5% CO_2. Cells from the second passage were used for all of the experiments and the culture medium was changed to serum-free DMEM for 48 hours before stimulation.

Percoll enrichment of adult mouse cardiomyocytes and non-cardiomyocytes

Adult mouse cardiomyocytes and cardiac fibroblasts were prepared from 10-week-old C57BL6 male mouse by the isolation of adult mouse cardiac myocytes from one heart AfSC procedure protocol [13]. After digestion, cells were dissociated, resuspended in differentiation medium, and loaded onto a discontinuous Percoll gradient as described previously [14]. Cardiomyocytes and noncardiomyocytes, which were mainly consisted of cardiac fibroblasts were separately collected, washed, and used for reverse transcriptional-polymerase chain reaction (RT-PCR).

RNA Extraction and RT-PCR Analysis

Total RNA was isolated by the guanidinium thiocyanate-phenol chloroform method. A total of 4 mg RNA was transcribed with MMLV reverse transcriptase and random hexamers. For PCR, the following primers from exon 15 and 17 of the murine G-CSFR were used: sense 5'-GTACTCTTGTCCACTACCTGT -3'; antisence, 5'-CAAGATACAAGGACCCCCAA -3' [15]. PCR was performed under the conditions included an initial denaturation at 94 °C for 2 minutes, followed by a cycle of denaturation at 94 °C for 1 minute, annealing at 58 °C for 1 minute, and extension at 72 °C for 1 minute. Sample was subjected to 40 cycles followed by a final extension at 72 °C for 3 minutes. The product was analyzed on a 1.5% ethidium bromide stained agarose gel.

Immunocytochemistry

Cardiomyocytes and non-cardiomyocytes from neonatal rats cultured on glass cover slides in DMEM with 10% FBS for 48 hours were incubated with or without anti G-CSFR (Santa Cruz Biotechnology) for 1 hour, then incubated with Cy3-labeled secondary antibodies according to manufacturer's direction. After wash, the cells were double-stained with florescein phalloidin (Molecular Probes) for 1 hour at room temperature.

Western blots

After appropriate treatments, cardiac fibroblasts were rinsed with ice-cold phosphate-buffered saline one time and frozen immediately in liquid nitrogen then stocked in –80 °C until use. Cells were by thawing in 150 ml of lysis buffer containing 25 mmol/L Tris-Hcl, 25 mmol/L Nacl, 0.5 mmol/L EGTA, 10 mmol/L Pyrophosphate, 1 mmol/L Na3VO4, 10 mmol/L NaF, 10 nmol/L Okadic acid, 1 mmol/L PMSF, 10 mg/ml Aprotinin, and 10 mg/ml Leupeptin. The extracts were centrifuged at 14000 rpm at 4 °C for 30 minutes, and the total protein concentration was measured with the BCA protein assay kit (Pierce). Proteins (30 μg) were separated in 8-10% SDS-PAGE and transferred onto a nitrocellulose transfer membrane (Schleicher & Schuell). After blocking in TBS-T (150 mmol/L NaCl, 50 mmol/L Tris, and 0.1% Tween 20, pH 7.4) containing 5% skim milk, membranes were probed with anti-phospho-PKCζ anti-phospho-ERK1/2, anti-phospho-Akt1 (cell signaling), anti-ERK2, anti-Akt1 (Santa Cruz

Biotechnology). ECL system (Amersham Biosciences, Inc.) was used for the detection.

Results

G-CSF Receptor Is Expressed on Cardiomyocytes and cardiac fibroblasts

To text whether G-CSFR is expressed on cardiomyocytes and cardiac fibroblasts in the adult heart, we performed a RT-PCR experiment by using specific primers for mouse G-CSFR. G-CSFR mRNA was detected in the adult cardiomyocytes and cardiac fibroblasts. The PCR product showed the expected size of 628 bp (Figure 1a). We also examined the G-CSFR expression in cultured rat cardiomyocytes and cardiac fibroblasts by immunocytochemistry. In agreement with a previous study of G-CSFR in living cells by using of G-CSFR / EGFP (enhanced green fluorescent protein) fusion constructs [16], immnostaining for G-CSFR revealed that G-CSFR localized to the plasma and cell membrane under stead-state conditions in cardiomyocytes and cardiac fibroblasts which was detected by the anti G-CSFR antibody. On the other hand, the signaling was disappeared when the anti G-CSFR antibody was deprived (Figure 1b, c).

G-CSF Induces Activation of Jak2/Stat3, PKCζ, ERK and Akt1 in Cultured Cardiac fibroblasts

We further examined whether activations Jak2/Stat3, PKCζ, ERK, and Akt1 were involved in G-CSF-induced signaling pathways in cultured cardiac fibroblasts. Cells were exposed to G-CSF (100 ng/ml) for 0, 0.5, 1, 2, 10, 15, and 30 minutes. Jak2/Stat3, PKCζ, ERK 1/2, and Akt1 activation were quantified by Western blot analysis. As shown in Figure 2, G-CSF significantly induced phosphorylation of Jak2/Stat3, PKCζ, ERK, and Akt1 in a time-dependent manner.

G-CSF downregulats protein levels of MMPs, collagen type I, and SM α-actin in Cultured Cardiac fibroblasts

We next examined whether MMPs, collagen, and SM α-actin protein levels were downregulated by G-CSF in cultured cardiac fibroblasts. We exposed the cells to G-CSF (100ng/ml) for 48 and 72 hours before preparation of whole cell protein lysates. Western blot analysis revealed that G-CSF significantly downregulated MMPs, collagen type I, and SM α-actin protein levels especially at 72 hours after G-CSF administration (Figure 3, 4).

Discussion

To our knowledge, we first demonstrated that G-CSFR was expressed on the cardiomyocytes and cardiac fibroblasts. G-CSF activated a series of kinase cascades including Jak2/Stat3, PKCζ, ERK, and Akt1 through G-CSFR in cultured cardiac fibroblasts, leading to down-regulation of MMPs, collagen type I, SM α-actin protein expression.

It has been recently reported that the combination therapy of G-CSF prevents cardiac dysfunction and remodeling after MI in mice [2]. The mobilization of bone marrow stem cells (BMSCs) to myocardium is considered to be a principal mechanism [17-19]. In this study, we showed that G-CSFR was expressed cardiomyocytes and cardiac fibroblasts. Suggesting that beneficial effects of G-CSF on the treatment of MI, at least in part, by direct exertion via G-CSFR on myocardium.

Previous studies reported that a MMP family was increased in the postinfarction heart failure models with permanent occlusion and large infarction and that the inhibitors beneficially affected cardiac remodeling and function [20-21], It is suggested that an increase in MMP has an aggravating effect on heart failure via collagen degradation. In the present study, we showed that MMPs protein expression were downregulated in G-CSF treated cells, this maybe a chain reaction when collagen synthesis was inhibited by G-CSF. Thus, by some possibility, downregulation of MMPs was one of the protective mechanism of G-CSF prevent from cardiac remodeling after MI. Suggesting that the beneficial effects of G-CSF, at least in part, by regulating the collagen degradation after MI. Furthermore, activation of Stat3 has been reported to promote neovascularization in the myocardium through enhanced expression of VEGF [22] and Akt1 pathway has been reported to play a critical role in cell survival and angiogenesis [23]. Our data show that G-CSF activated Jak2/Stat3, PKCζ, ERK 1/2, and Akt1 in a time-dependent manner.

In summary, we demonstrated for the first time of how G-CSF exerts its effect on cardiac fibroblasts in vitro. G-CSF may preserve cardiac function not only by promoting mobilization of BMSCs into the myocardium but also by acting directly on cardiomyocytes and cardiac fibroblasts through its receptor. But it remains unclear whether G-CSF induced increase in VEGF expression in cardiac fibroblasts indeed

contributes to the neovascularization of MI heart. Further studies are needed to elucidate whether G-CSF have the same effects in vivo.

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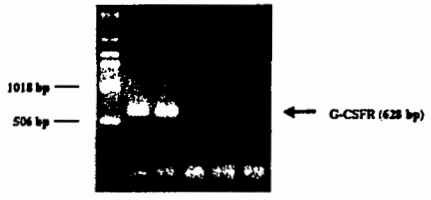
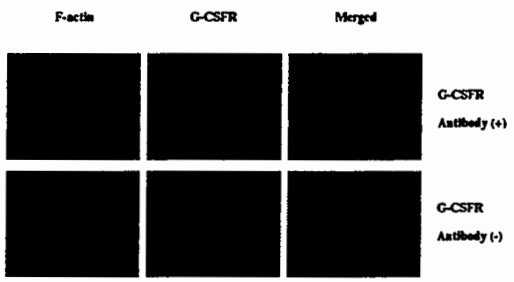
Figure 1a



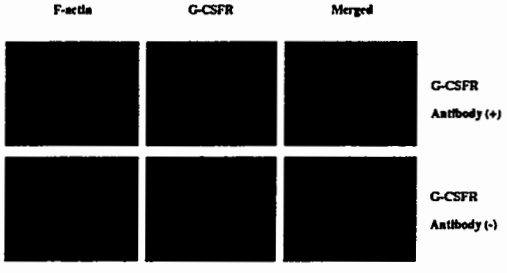
1. Cardiac myocytes (MI mice)
2. Non Cardiac myocytes (MI mice)
3. Cardiac myocytes (sham)
4. Non Cardiac myocytes (sham)
5. Negative control

Figure 1b



cardiomyocytes

Figure 1c



cardiac fibroblasts

Figure 2

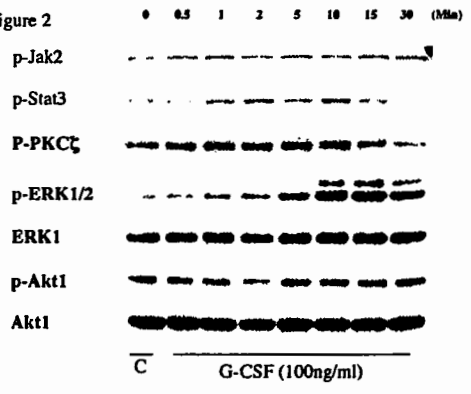


Figure 3

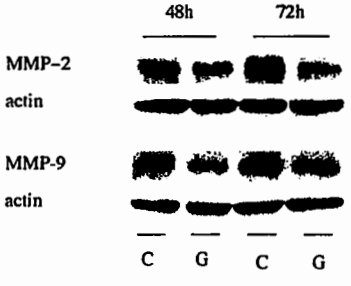


Figure 4