

Granulocyte colony-stimulating factor activates Wnt signal to sustain gap junction function through recruitment of β -catenin and cadherin.

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Introduction

Treatment of patients with chronic heart failure has been an area of great interests, because the prognosis in heart failure remains to be poor despite novel therapeutic modalities. Among various causes in heart failure, myocardial infarction is the most common and novel therapeutic modalities for heart failure are much needed. Researchers have recently focused on the novel therapeutic field of cardiac regeneration induced by transplantation of embryonic stem cells, mesenchymal cells, bone marrow cells, or myoblasts and the administration of granulocyte-colony stimulating factor (G-CSF).

Previous studies have shown that cardiomyocytes are efficiently and remarkably transdifferentiated from transplanted progenitor cells derived from bone marrow cells. However, recent studies have revealed that the regeneration rate from progenitor cells to cardiomyocytes is quite low and they frequently fuse with resident cardiomyocytes, and that the actual transdifferentiation rate is extremely low not enough to improve the entire cardiac function after the regeneration therapy if transdifferentiation alone could be involved in the situation [1–4]. Surprisingly, however, such treatments in fact improve the function by unknown mechanisms. Even with treatments using G-CSF, it has been also reported that the transdifferentiation rate is quite low, but that cardiac function is markedly improved [5,6]. Recently it has been reported that G-CSF has a direct effect on cardiomyocytes through the receptor responsible for cardiac hypertrophy and inhibits both apoptosis and remodeling in the failing heart following myocardial infarction [7].

It is important to consider two of key factors affecting survival rate in patients with chronic heart failure, i.e., suppression of the progression of cardiac remodeling and suppression of the fatal arrhythmia. As an elegant previous study demonstrates, G-CSF inhibits cardiac remodeling, resulting in survival rate improvement [7]. However, it remains to be investigated whether G-CSF has an anti-arrhythmia effect, because the study referred to did not investigate this in detail. Furthermore, there are no reports that reveal the detailed molecular mechanisms of the anti-arrhythmia effect of G-CSF.

Connexin (Cx) 43, a component of the gap junction complex, has recently been reported to be involved in modulating arrhythmia, The survival of Cx43 knockout mice was lower than that of wild-type mice because of greater susceptibility to lethal arrhythmia, including ventricular tachycardia and fibrillation. This suggests that Cx43 plays a crucial role in modulating arrhythmia [8–11]. We recently demonstrated in rats that acetylcholine (ACh) activates the function of gap junctions by sustaining the Cx43

protein level leading to inhibition of the lethal arrhythmia that follows myocardial infarction [12].

In the present study, we assessed the hypothesis that G-CSF has an anti-arrhythmia effect apart from its anti-apoptotic effect, through the sustainable localization of Cx43 on the plasma membrane, leading to an additive contribution to the increased survival rate following the ischemic heart diseases.

Materials and Methods

Cell Culture

To examine the effect of G-CSF on cardiomyocytes, we used primary cardiomyocytes isolated from neonatal rats and H9c2 cells. H9c2 cells are frequently used to investigate signal transduction and channels instead of rat primary cardiomyocytes. H9c2 cells have an advantage to be more efficiently gene-transferred than primary cardiomyocytes, therefore, in the present study H9c2 cells were used especially for a reporter assay. H9c2 cells were incubated in DMEM containing 10% FBS and antibiotics. Rat primary cardiomyocytes were isolated from 2- to 3-day-old neonatal Wistar rats and incubated in DMEM/Ham F-12 containing 10% FBS and ITS supplement. HEK293 cells were used in gene transfection studies; they were cultured in DMEM containing 10% FBS.

Western Blot Analysis

Rat primary cardiomyocytes or H9c2 cells were treated with 10 ng/ml of G-CSF to evaluate their protein expression level under normoxic or hypoxic conditions with 1 mM of cobalt chloride (CoCl₂) or an oxygen concentration of 1%. In the case of hypoxia, cardiomyocytes were pretreated with G-CSF 24 h before hypoxia, followed by 12 h of hypoxic treatment. Cell lysates were mixed with a sample buffer, fractionated by SDS-PAGE and transferred onto membranes. The membranes were incubated with polyclonal primary antibodies against G-CSF receptor, phospho-JAK2 (Santa Cruz Biotechnology, Santa Cruz, CA), β -catenin (Cell Signaling Technology, Beverly, MA) or Cx43 (ZYMED Laboratories Inc., South San Francisco, CA), and monoclonal antibodies against α -tubulin (Lab Vision, Fremont, CA), phospho-Akt (Cell Signaling Technology, Beverly, MA, USA) or pan-cadherin (Sigma Chemical Co., St. Louis, MO). They were then reacted with an HRP-conjugated secondary antibody (BD Transduction Laboratories, San Diego, CA, USA, and Promega, Madison, WI). Positive signals were detected with an enhanced chemiluminescence system (Amersham, Piscataway, NJ). In each study, experiments were performed in duplicate and repeated four or five times (n=4 or 5). Only representative data alone are shown.

MTT Activity Assay

To evaluate the effects of hypoxia on the mitochondrial function of cardiomyocytes, we measured the reduction activity of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) in cardiomyocytes under hypoxic conditions in the presence or absence of G-CSF. The cells were pretreated with 10 ng/ml G-CSF for 24 h and then subjected to 12-h hypoxia. One hour before sampling, the MTT reagents were added to the culture

and incubated; and absorbance at 450 nm was measured (Cell Counting Kit-8; Dojindo, Kumamoto, Japan).

Myocardial infarction in rats

Left ventricular myocardial infarction was induced by left coronary artery ligation in anesthetized male Wistar rats, which were ventilated artificially. G-CSF (5 µg/kg) was administered intraperitoneally 24 h before coronary ligation. For 30 min after the coronary ligation, ECG was recorded to evaluate arrhythmia, and blood pressure was also monitored in rats with or without G-CSF treatment (no treatment, n=4; G-CSF treatment, n=7).

Transfection

To investigate whether G-CSF regulates Wnt signaling, the reporter vector of TOPflash (Upstate Cell Signaling Solutions, Lake Placid, NY) was used for transfection into H9c2 cells. The reporter vector possesses three copies of the T-cell factor binding site. H9c2 cells were transfected together with TOPflash vector and SeaPansy vector by Effectene (Quiagen, Valencia, CA) according to the manufacturer's protocol. After 24 h of transfection, G-CSF (10 ng/ml) was added. Transfected cells were lysed 24 h later, and the luciferase activity was evaluated with a dual luciferase activity assay kit from Wako Chemical (Tokyo, Japan).

Immunocytochemistry

Rat primary cardiomyocytes were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.1% Triton X-100 for 10 min. Then, cells were incubated with 4% skim milk and successively incubated with an anti-Cx43 polyclonal antibody (ZYMED Laboratories Inc.), anti-β-catenin polyclonal antibody (Cell Signaling Technology) or anti-pan-cadherin monoclonal antibody (Sigma Chemical Co.), in 1% skim milk at 4°C overnight, followed by FITC or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) at 4°C overnight. After mounting, imaging of the immunoreactivities were examined and obtained with an OLYMPUS FLUOVIEW 300 laser-scanning confocal microscope (OLYMPUS, Tokyo, Japan).

RT-PCR

Total RNA was isolated from primary cardiomyocytes according to a modified acid

guanidinium-phenol-chloroform method using an RNA isolation kit (ISOGEN; Nippon Gene, Tokyo, Japan) and was reverse-transcribed to obtain a first-strand cDNA. This first-strand cDNA was amplified by specific primers, and the PCR products were fractionated by electrophoresis.

Functional analysis of gap junctions using a scrape/scratch or microinjection method

A scrape-loading method was used to introduce macromolecules into cultured cells by inducing a transient tear in the plasma membrane, thereby allowing sensitive determination of cell-cell communication. Following treatment with 10 ng/ml G-CSF for 24 h, 1% Lucifer Yellow was applied to the center of the coverslip, on which cardiomyocytes were cultured. A 27-gauge needle was used to create two longitudinal scratches through the cell monolayer. One minute later, the cardiomyocytes were washed and finally examined by Axiovert fluorescence microscopy (Carl Zeiss, Inc.). Lucifer Yellow is transmitted from one cell to another, presumably across patent gap junctions [13–16]. The area of the dye transferred from the scratched margin in hypoxia or hypoxia with G-CSF treatment was semiquantified using the NIH image system and compared with that in normoxia. In another dye transfer assay using a microinjection method, LY was injected electrophoretically via a conventional microelectrode made from glass capillaries using micromanipulator system (SURUGA SEIKI Co, Ltd., Shizuoka, Japan). The outer diameter of microelectrodes was 0.15 to 0.25 μm . An inward square-pulse current of 100 msec. duration was injected at 2 Hz for 1 minute using a voltage pulse generator (SEN7203, NIHON KONDEN, Tokyo, Japan). In this microinjection, experiments could not be practically conducted in a hypoxic chamber, therefore, a chemical hypoxic reagent of CoCl_2 (1 mM) was used to induce cellular hypoxia.

Electrophysiological evaluation of the G-CSF effect on cardiomyocytes during hypoxia using mitochondrial membrane potential dye

Rat primary cardiomyocytes were pretreated with 10 ng/ml G-CSF for 24 h, followed by 1% hypoxia for 1 h. During hypoxia, the cardiomyocytes were incubated with 2 μM di-4-ANEPPS

(pyridinium,4-{2-[6-(dibutylamino)-2-naphthalenyl]ethenyl}-1-(3-sulfopropyl)-hydroxide) for 15 min followed by washing. In the presence of cytochalasin D (5 μM), during hypoxic conditions with or without G-CSF, the cardiomyocytes were observed to evaluate the propagation of action potentials, using an imaging system for opto-electrophysiology, MiCAM02 (Brainvision, Tokyo, Japan).

Densitometry

The western blotting data were analyzed using Kodak 1D Image Analysis Software (Eastman Kodak Co., Rochester, NY).

Statistics

The data are presented as means \pm S.E (standard error). Mean values between two groups were compared using the unpaired Student's t test. Multiple comparisons of results were assessed by ANOVA for multiple comparisons of results. Differences were considered significant at $P < 0.05$.

Results

G-CSF transduces the signal through its receptor in cardiomyocytes

The expression level of G-CSF receptor (G-CSFR) was examined by Western blot analysis. As shown in Figure 1, G-CSFR was detected in rat primary cardiomyocytes with a molecular weight of 130 kDa. To investigate whether the G-CSFR was functional in cardiomyocytes, we evaluated signal transduction through JAK2 phosphorylation, one of the major signaling molecules for G-CSFR. As shown in Figure 1, G-CSF (10 ng/ml) increased JAK2 phosphorylation in cardiomyocytes. Likewise, JAK2 was also phosphorylated in H9c2 cells, cell lines of rat ventricular myocytes, by G-CSF suggesting that H9c2 cells are equipped with the similar machinery responding G-CSF. As a result, along with the JAK2 phosphorylation, an important signal responsible for cardiac hypertrophy, G-CSF actually increased cell size within 24 h ($365.2 \pm 22.2\%$, # $P < 0.01$ vs. control, n=6) comparably with 1 μ M ET-1 ($287.2 \pm 35.3\%$, # $P < 0.01$ vs. control, n=6). A similar effect was also observed in H9c2 cells (data not shown). Although a G-CSFR-specific receptor antagonist is not available, taken together with the fact that JAK2 is known to be a G-CSFR-related molecule, it is suggested that G-CSF acts on cardiomyocytes through its receptor.

G-CSF has a cell survival effect on cardiomyocytes during hypoxic condition

To study whether G-CSF has a cell survival effect on rat primary cardiomyocytes, we first examined Akt phosphorylation, a cell survival signal, in response to G-CSF. As demonstrated in Figure 2, Akt was phosphorylated by G-CSF (10 ng/ml) within 4 h, suggesting that G-CSF plays a role in transducing a cell survival signal. To investigate whether G-CSF actually possesses such a cell-protective effect, cardiomyocytes were treated with 1 mM CoCl₂, a hypoxic reagent, for 8 h following the 24-h G-CSF pretreatment. With CoCl₂ alone, cell viability, which was evaluated with the MTT reduction assay, was markedly reduced in the absence of G-CSF. However, the G-CSF pretreatment sustained cell viability even in the presence of CoCl₂ (n=6, $P < 0.001$ vs. CoCl₂) for 8 h. This suggests that G-CSF has a cell-protective effect.

G-CSF shows an anti-arrhythmia effect on acute myocardial infarction

It has been reported that G-CSF has a chronic effect on myocardial infarcted rats, leading to an increase in the survival rate of rats with chronic myocardial infarction, and it has been speculated that this effect probably occurs through inhibition of cardiac remodeling, i.e. inhibition of death due to chronic cardiac pump failure. However, the

effects of G-CSF on suppression of fetal arrhythmia in the chronic stage were not fully investigated. Furthermore, an acute effect of G-CSF on the infarcted heart has not yet been profoundly studied, especially the effect of G-CSF on arrhythmogenesis in acute myocardial infarction. Therefore, we performed an *in vivo* study to investigate whether G-CSF pretreatment has an anti-arrhythmia effect on the myocardial infarcted heart.

Rats were pretreated or not pretreated with 5 $\mu\text{g}/\text{kg}$ G-CSF 24 h before the left coronary artery ligation. Without G-CSF treatment, all rats suffered from ventricular arrhythmia. As demonstrated in Figure 3A, a G-CSF-untreated rat abruptly showed a decrease in blood pressure due to ventricular fibrillation (VF), however, the treated rat sustained blood pressure during the entire time course. Especially, VF was usually observed within 30 minutes after the coronary ligation (100%), with a lethal episode in some rats because of sustained VF. In contrast, the G-CSF-pretreated rats suffered less frequently from ventricular arrhythmia. Specifically, VF was never detected in the G-CSF-treated rats (0%). In rats treated with G-CSF, the phosphorylated forms of Cx43 protein in the hearts was greatly increased, compared with those non-treated hearts (Figure 3A). Furthermore, G-CSF also reduced the other arrhythmia parameter, the duration of ventricular tachycardia (VT) (10.9 ± 2.3 vs. 58.7 ± 3.4 sec, $P < 0.01$). These results suggest that G-CSF is involved in inhibiting arrhythmia induced by myocardial infarction.

G-CSF activates Wnt signal in cardiomyocytes

To further clarify the anti-arrhythmia mechanism of G-CSF, we first focused on the Wnt signal, which is involved in cell-cell communication as well as cell survival. Therefore, the Wnt signal was examined in cardiomyocytes in response to G-CSF. As shown in Figure 4A, H9c2 cells were transfected with the TOPflash luciferase vector and SeaPansy vector for dual luciferase evaluation system. Luciferase activity was increased by G-CSF up to 10.1- and 25.9-fold following the 24- and 36-h treatments, respectively ($\#P < 0.01$ vs. 0h). In accordance with this result, β -catenin protein expression was greatly increased by G-CSF in whole cell lysates from both H9c2 cells and primary cardiomyocytes (292.6 ± 14.4 %, $\#P < 0.01$ and 437.0 ± 10.3 %, $\#P < 0.01$, respectively) (Figure 4B). Since β -catenin is known to be regulated by a negative regulator of Wnt signal GSK-3 β , which phosphorylates β -catenin and accelerates degradation of the protein, it is suggested that G-CSF activates the Wnt signal in cardiomyocytes, resulting in inhibition of β -catenin degradation.

G-CSF inhibits degradation of Cx43

According to our results, we speculated that G-CSF plays an important role in inhibiting the degradation of protein other than β -catenin through Wnt signal activation. Therefore, we examined whether G-CSF increased the Cx43 protein level. Cx43 is also known to possess a shorter protein half-life and rapidly regulated by the protein degradation system.

Initially Cx43 mRNA expression level was evaluated by RT-PCR. As shown in Figure 5, the Cx43 mRNA level was not increased by G-CSF. In contrast, the total Cx43 protein level was markedly increased by G-CSF in rat primary cardiomyocytes. Specifically, a Cx43 phosphorylated form, which was composed of upper two bands designated as P-Cx43 in Figure 5, was more increased by G-CSF. The lower band designated as a non-phosphorylated band (N-Cx43 in Figure 5) was also up-regulated by G-CSF.

To further elucidate the effect of G-CSF on the localization of gap junction components on the cell surface, an immunocytochemical study was conducted using antibodies against β -catenin, pan-cadherin and Cx43 and it was also investigated whether the components had any mutual interaction. Such G-CSF-increased β -catenin signals overlapped, especially on the plasma membrane, with those of Cx43 (Figure 6), and it was colocalized with increased signals of cadherin, which was also evident by Western blot analysis (Figure 6), suggesting that G-CSF increases protein levels of gap junction components, including β -catenin, cadherin, and Cx43, and that the localization of these components on the cytoplasmic membrane is tightly regulated by G-CSF.

G-CSF functionally activates the gap junction in primary cardiomyocytes

To further study whether G-CSF modulates the function of gap junctions, a dye transfer study was conducted using Lucifer Yellow with a scrape/scratch or microinjection method. Hypoxia decreased the gap junction function along with reduction in dye transfer, compared with normoxia, demonstrating that gap junction was impaired by 1% of hypoxia (Figure 7A). However, pretreatment with G-CSF inhibited the reduction of dye transfer, and the transfer level during hypoxia was sustained at a level similar to that in normoxia by G-CSF (Figure 7A). A microinjection method also indicated that hypoxia induced by 1 mM of CoCl_2 decreased dye transfer, resulting in the dye remaining in the cell. In this microinjection, chemical hypoxic reagent CoCl_2 was used instead of 1% hypoxia, because microinjection could not be practically performed in a hypoxic chamber. However, G-CSF treatment resulted in recovery of cell-cell communication even during hypoxia (Figure 7B). This suggests that G-CSF regulates gap junction to enhance cell-cell communication through the stabilization of gap

junction components to the plasma membrane. This may contribute to the anti-arrhythmia effect.

To investigate the electrophysiological effects of G-CSF on gap junctions, the propagation of membrane potential in cardiomyocytes was evaluated using Di-4-ANEPPS

(pyridinium,4-{2-[6-(dibutylamino)-2-naphthalenyl]ethenyl}-1-(3-sulfopropyl)-hydroxide). As demonstrated in Figure 8, in the absence of G-CSF, the activation and propagation of the action potential was dampened by hypoxia. In contrast, in G-CSF-pretreated cardiomyocytes, activation and propagation of the membrane potential were sustained even in hypoxia, suggesting that G-CSF sustains gap junction function even during hypoxia.

Taken these results together, we suggest that G-CSF inhibits the impairment of the gap junction function during hypoxia by sustaining the protein levels of the components in cardiomyocytes. Therefore, the use of G-CSF could lead to an anti-arrhythmia effect on cardiomyocytes during hypoxia.

Discussion

In contrast with the previous studies using G-CSF, the present study demonstrates a novel function of G-CSF. We demonstrate that G-CSF stabilizes the gap junction complex through the Wnt signal to sustain its function even under hypoxic conditions. Therefore, we suggest that G-CSF inhibits impairment of the gap junction function and has the potency to suppress ventricular arrhythmia after acute myocardial ischemia and may be used as a therapeutic tool.

In the present study, we initially demonstrate that G-CSF has a direct effect on cardiomyocytes through G-CSFR with activation of JAK2 signal transduction, as reported in previous studies [7], and that G-CSF also plays a pivotal role in increasing the protein expression levels of β -catenin and Cx43. β -catenin is known to be involved in regulating the gap junction complex and other membrane-associated molecules including cadherin. These are also known to be indispensable for gap junction complex formation [17–21]. In accordance with this, the Wnt signal is activated by G-CSF, as demonstrated by TOPflash reporter assay. Activation of the Wnt signal, resulting in increased β -catenin levels, as found in this study, is also reported to be responsible for cell survival [22–24]. It also reveals that G-CSF protects cells against chemical hypoxic stress. The results can be combined to suggest that G-CSF plays a role in cardioprotection through the survival pathway of the Wnt signal.

Furthermore, this study reveals a novel G-CSF function acting through β -catenin. Besides the cell survival signal, G-CSF increases cell-cell communication by inhibiting Cx43 protein degradation and increasing the cadherin level as shown in Figure 6, resulting in increased expression levels of gap junction components. These effects are confirmed by the sustained dye transfer in cardiomyocytes even during hypoxia in the presence of G-CSF alone, as shown in scrape/scratch and microinjection experiments. These *in vitro* data are also confirmed by a rat model of acute myocardial ischemia with a reduced frequency of ventricular tachycardiac arrhythmia after G-CSF treatment. Therefore, from the results of the present study, we suggest that G-CSF has another novel effect of anti-arrhythmia through the activation of gap junction function.

Many studies have been performed on the pathogenesis of arrhythmia [25-27]. Recently the impairment of gap junction function has been focused on as one of the pathogenic features [8-11]. Since heterogeneity of conduction velocity is induced by hypoxia through impairment of gap junctions, it is considered that impairment of gap junctions is responsible for one of the arrhythmogenic substrates. Specifically, Cx43 knockout mice are reported to be remarkably susceptible to ventricular arrhythmia,

causing sudden death [9]. In addition, our recent works have revealed that acetylcholine plays a pivotal role in sustaining the Cx43 protein level during hypoxia and that vagal nerve stimulation inhibits arrhythmia induced by acute myocardial infarction by sustaining the Cx43 protein level [12,28]; this suggests that intact gap junction function is necessary for the inhibition of arrhythmia. Based on these studies, it is suggested that a drug that increases the Cx43 protein expression level or sustains its appropriate localization might provide a therapeutic tool for arrhythmia in addition to currently available anti-arrhythmia drugs.

The present study suggests a precise mechanism for the regulation of Cx43 protein level by G-CSF, which activates Wnt signal, leading to stabilization of the protein localization on plasma membrane along with other membrane-associated molecules and to improvement of gap junction function. There are no studies clearly demonstrating a precise link between G-CSF, Wnt signal and the anti-arrhythmia effect, therefore, the present study was performed. However, several previous studies suggest each link, i.e. Wnt signal and Cx43. The present results are compatible with the study demonstrating that activation of the Wnt signal by lithium chloride in cardiomyocytes causes an increase in the Cx43 protein level [29]. Other studies also reveal that the activated Wnt signal increases cell aggregation, which is resistant to agitation, suggesting an increase in intercellular communication [30,31]. These findings indicate that Wnt signal activation accelerates cell-cell interaction through molecules involved in gap junction formation. Gap junctions are composed of connexin subtypes and are supported by the formation of complexes with cadherin and β -catenin [32,33]. β -catenin is located in the subcytoplasmic membrane and anchors Cx43 to the membrane [29,30]. It is reported that the Cx43 function is modulated by the total protein level as well as the phosphorylation level [34] and is also affected by these anchoring molecules. These are tightly associated with Cx43, resulting in complex formation, and are essential in maintaining the gap junction function, because knockout mice, including cadherin knockout mice, show abnormal gap junction function, causing susceptibility to arrhythmia [17]. Heterogeneous cadherin knockout mice do not survive as long as wild-type mice, because of sudden death; Cx43 localization is also impaired and it is not targeted to the plasma membrane, resulting in impairment of the function [17].

The present study reveals a novel function of G-CSF and presents a new therapeutic insight into arrhythmia. These are based on our results showing that G-CSF activates the Wnt signal, and that the Cx43 protein level is sustained during hypoxia by G-CSF. Furthermore, the expression levels of Cx43, cadherin, and β -catenin are all increased by G-CSF, as is their appropriate localization at the plasma membrane. These results

suggest that G-CSF regulates these protein levels and their localization, thereby activating their function, and prompts us to speculate that G-CSF actually possesses the anti-arrhythmia effects.

In vivo studies indicate an anti-arrhythmia effect on the acute ischemic heart. Surprisingly, G-CSF efficiently decreases the incidence of lethal ventricular tachycardia in rats with myocardial infarction. G-CSF clearly decreases the duration of sustained ventricular tachycardia. These effects of G-CSF might contribute to the increased survival of rats with acute myocardial infarction, as reported in a recent study [35], which only suggests a link between G-CSF and an anti-arrhythmia effect alone, but does not reveal the precise mechanisms. Based on other studies using myocardial infarcted rats, their findings suggest that G-CSF inhibits cardiomyocytes apoptosis during the progression of heart failure [7], accelerates angiogenesis [36], and enhances the attenuation of the inflammatory reaction during the acute myocardial infarction [5]. Although those speculative aspects of G-CSF are not independent, the new effect of G-CSF revealed by the present study may indicate a pivotal role for G-CSF in inhibiting arrhythmia by modulating gap junction components. Therefore, our study may provide a novel therapeutic indication for the use of G-CSF in patients with myocardial infarction in order to inhibit lethal arrhythmia in the acute phase.

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Figure legends

Figure 1

G-CSF receptor protein expression is detected in rat primary cardiomyocytes and transduces the signal through JAK2 phosphorylation.

G-CSF receptor was expressed in rat primary cardiomyocytes (A), and G-CSF (10 ng/ml) phosphorylated JAK2 within 4 hours, one of crucial factors responsible for cardiac hypertrophy in rat primary cardiomyocytes and H9c2 cells. Representative data are shown from four independent experiments (n=4 in each cell) (B). The G-CSF treatment was accompanied by enlargement of cell size ($\#P < 0.01$ vs. control, n=6), the extent of which was comparable with that of ET-1 (1 μ M) ($\# P < 0.01$ vs. control, n=6). Bars in the upper panels, 10 μ m; lower panels, 20 μ m (C).

Figure 2

G-CSF induces Akt phosphorylation and protects cardiomyocytes from a hypoxic mimetic.

Akt was phosphorylated by G-CSF (10 ng/ml) with a longer time course. Representative data are shown from four independent experiments. G-CSF-pretreated cardiomyocytes (24 h) showed comparable cell viability even with 1 mM CoCl₂ for 8 h, evaluated by the MTT reduction assay (n=6).

Figure 3

G-CSF inhibits the occurrence and frequency of ventricular arrhythmia induced by acute myocardial infarction.

A representative time course of blood pressure with or without the G-CSF pretreatment is shown. In rats without G-CSF pretreatment, VF occurred in the late phase after coronary ligation, causing abrupt reduction of blood pressure, but spontaneously attenuated (n=6). The occurrence of ventricular fibrillation (VF) was 100% in the non-treated rat. In contrast, G-CSF-treated rats did not show VF (n=7). Furthermore, G-CSF increased a phosphorylated Cx43 expression level in the heart. Representative Western blot data are shown (1-3, no treatment; 4-6, G-CSF-treatment) (A). In accordance with this, G-CSF also decreased the duration of ventricular tachycardia (10.9 ± 2.3 vs. 58.7 ± 3.4 sec., $P < 0.01$) (B).

Figure 4

G-CSF activates the Wnt signal in cardiomyocytes through the increased β -catenin level

with a slow time course.

The luciferase activity of TOPflash vector, adjusted by a SeaPansy luciferase activity, was increased by 10 ng/ml G-CSF in cardiomyocytes. The G-CSF-induced increase in the luciferase activity was evident within 24 h ($\#P < 0.01$ vs. 0h). Representative data from four independent experiments are shown (A). The increased luciferase activity contributed to elevated β -catenin protein expression (n=4). Both H9c2 cells and primary cardiomyocytes increased β -catenin protein expression ($\#P < 0.01$ vs. 0h in each cell group) (B).

Figure 5

The Connexin43 (Cx43) protein expression level is increased by G-CSF, independent of the transcriptional regulation, under the normoxic conditions.

The Cx43 mRNA level was not affected by 10 ng/ml G-CSF within 24 h; however, the protein expression level including upper two phosphorylated forms (P-Cx43) and lower one (N-Cx43) was increased by G-CSF within 24 h (n=5). In Western blot analysis Cx43 is known to be composed of upper bands as phosphorylated forms and lower band as non-phosphorylated one.

Figure 6

G-CSF increased immunoreactivities of β -catenin, cadherin, and Cx43 and increased their colocalization under normoxic conditions.

Primary cardiomyocytes from G-CSF-treated rats showed more immunoreactivities of β -catenin, cadherin, and Cx43, compared with non-treated cardiomyocytes, as also shown with increased expression of cadherin in Western blot analysis. These immunoreactivities overlapped especially in the cytoplasmic membrane. Compared with control, distribution of β -catenin was specifically detected on cytoplasmic membrane in G-CSF-treated cardiomyocytes. Representative data from six independent experiments are shown (n=6). Bars, 20 μ m.

Figure 7

G-CSF enhances the cell-cell communication as demonstrated by the scrape/scratch and microinjection methods.

Lucifer yellow dye was not efficiently transferred during 1% hypoxia, evaluated by a scrape/scratch method (A), and treatment with 1 mM of CoCl_2 , evaluated by a microinjection method (B), compared with normoxia. However, G-CSF pretreatment sustained dye transfer at a level similar to that during normoxia (n=5) (A, B)

Figure 8

Propagation of membrane potential in rat primary cardiomyocytes is sustained by G-CSF during hypoxia.

The propagation of the action potential, detected by di-4-ANEPPS, in rat primary cardiomyocytes decreased during hypoxia, with a shorter action potential duration and lower frequency. However, in G-CSF-pretreated cardiomyocytes the action potential propagated comparably with normoxic cardiomyocytes. Duration and frequency were not remarkably decreased by hypoxia in G-CSF-treated cardiomyocytes. Representative data from five independent experiments are shown (n=5).

Kuwabara
Figure 1

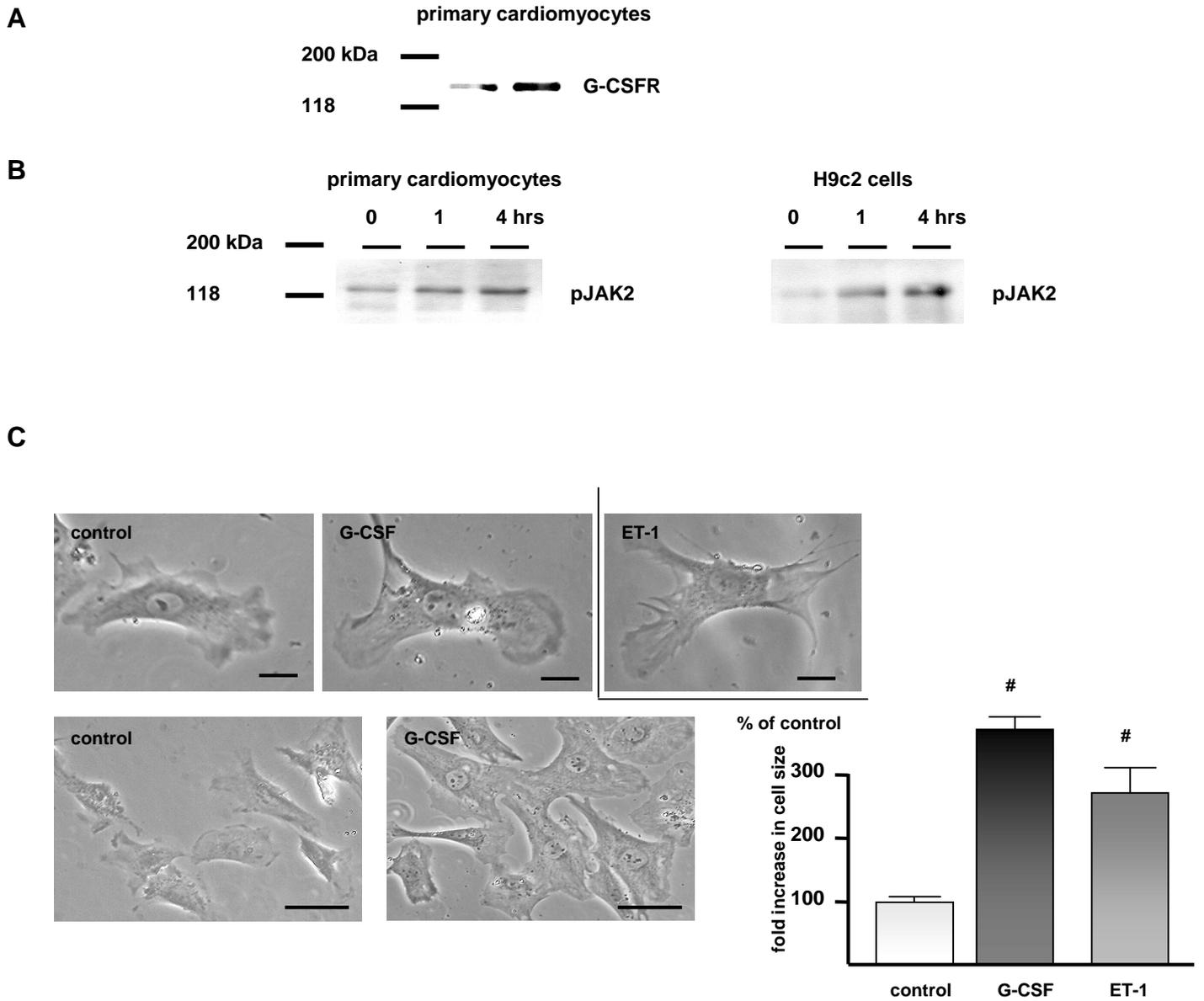
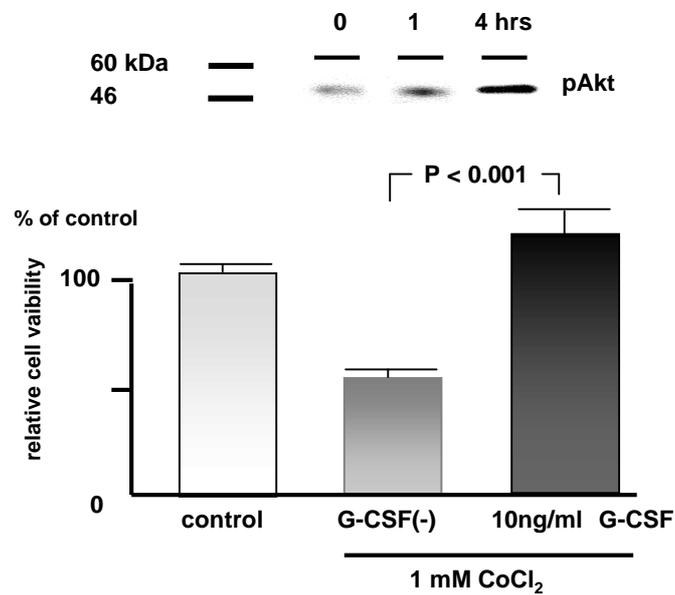


Figure 2



Kuwabara
Figure 3

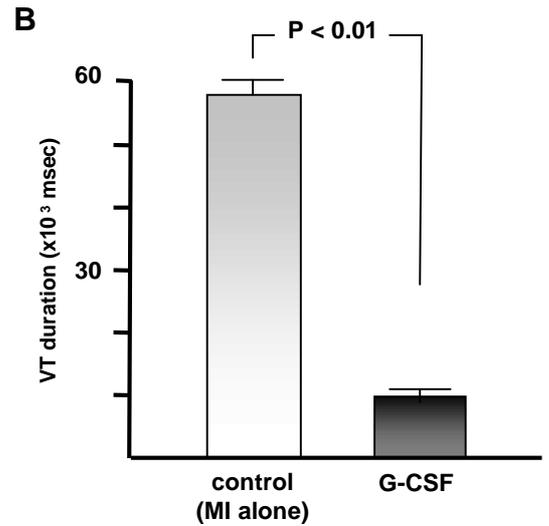
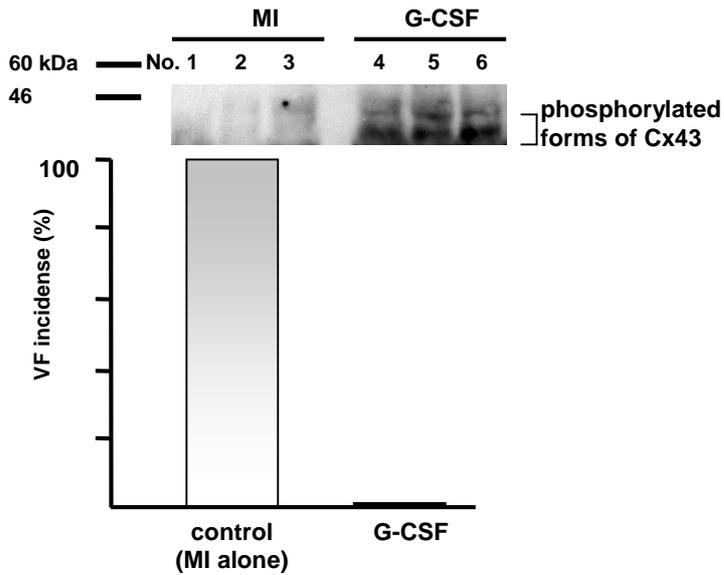
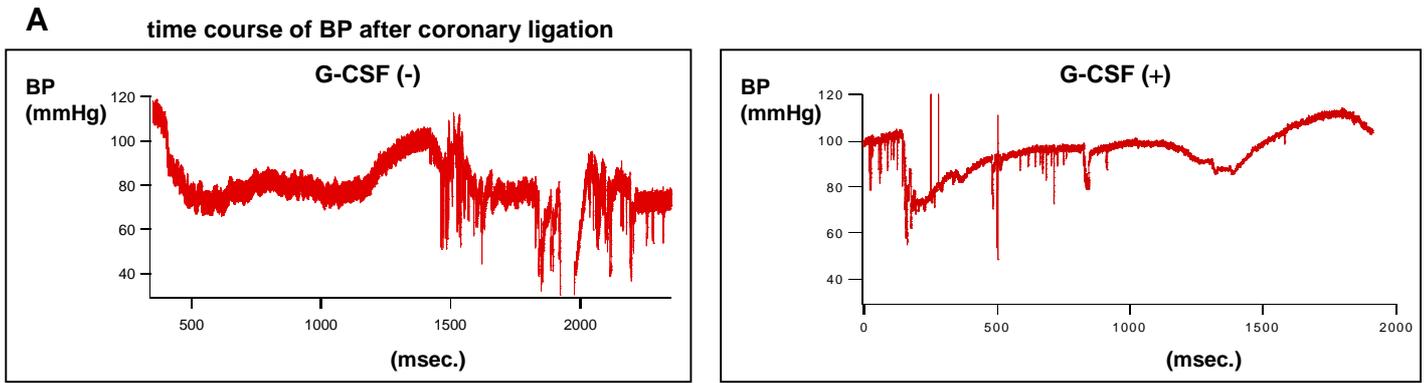
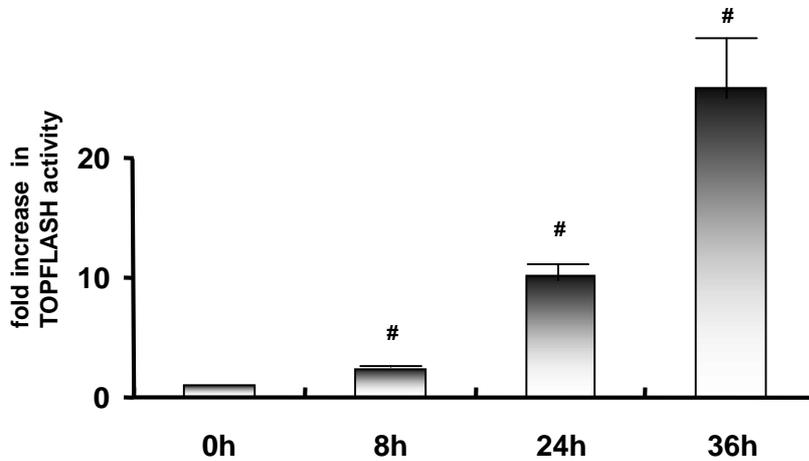
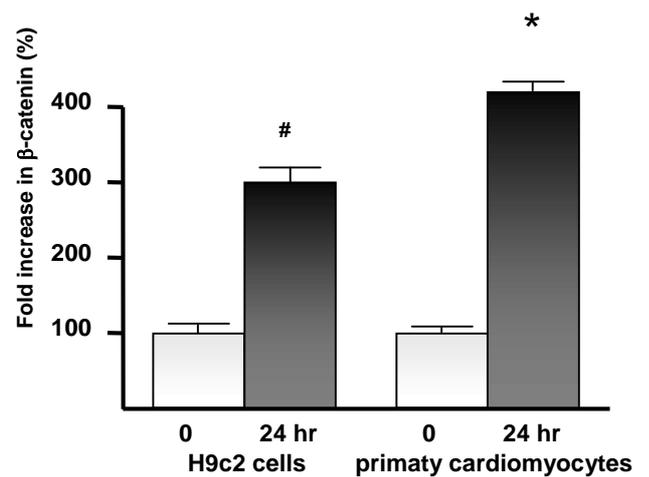
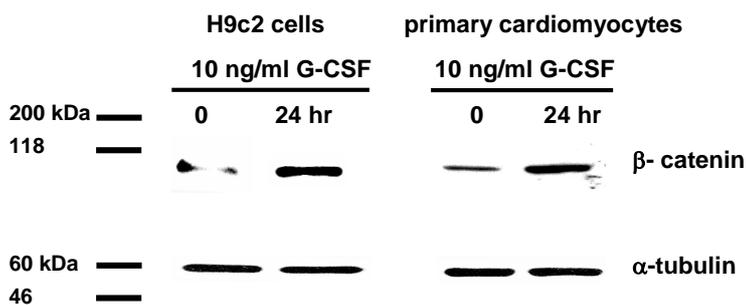


Figure 4
A



B



Kuwabara
Figure 5

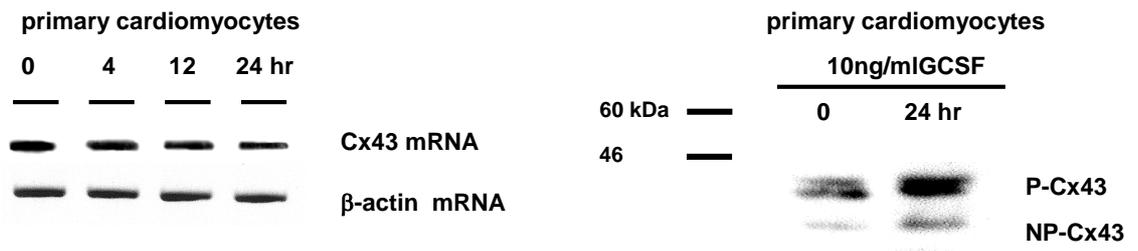
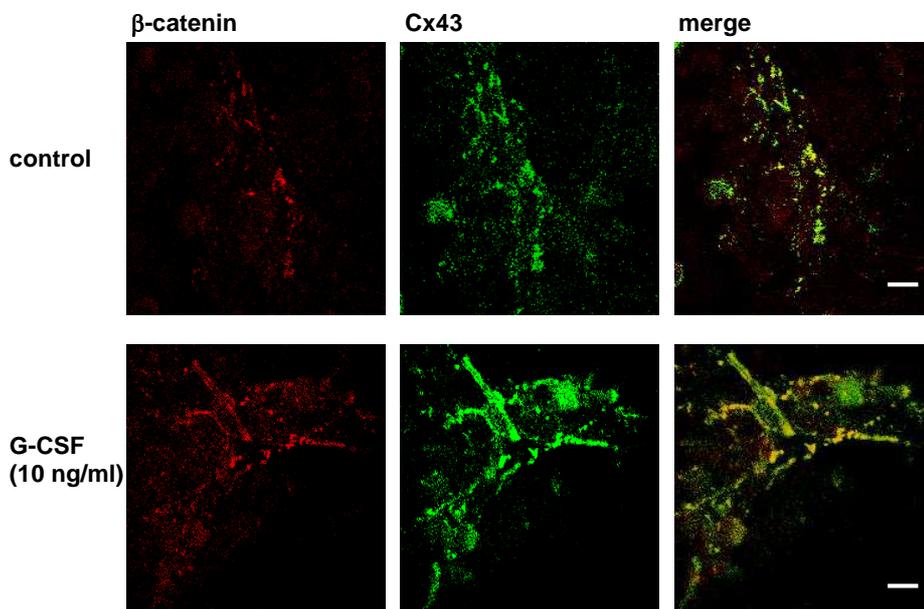
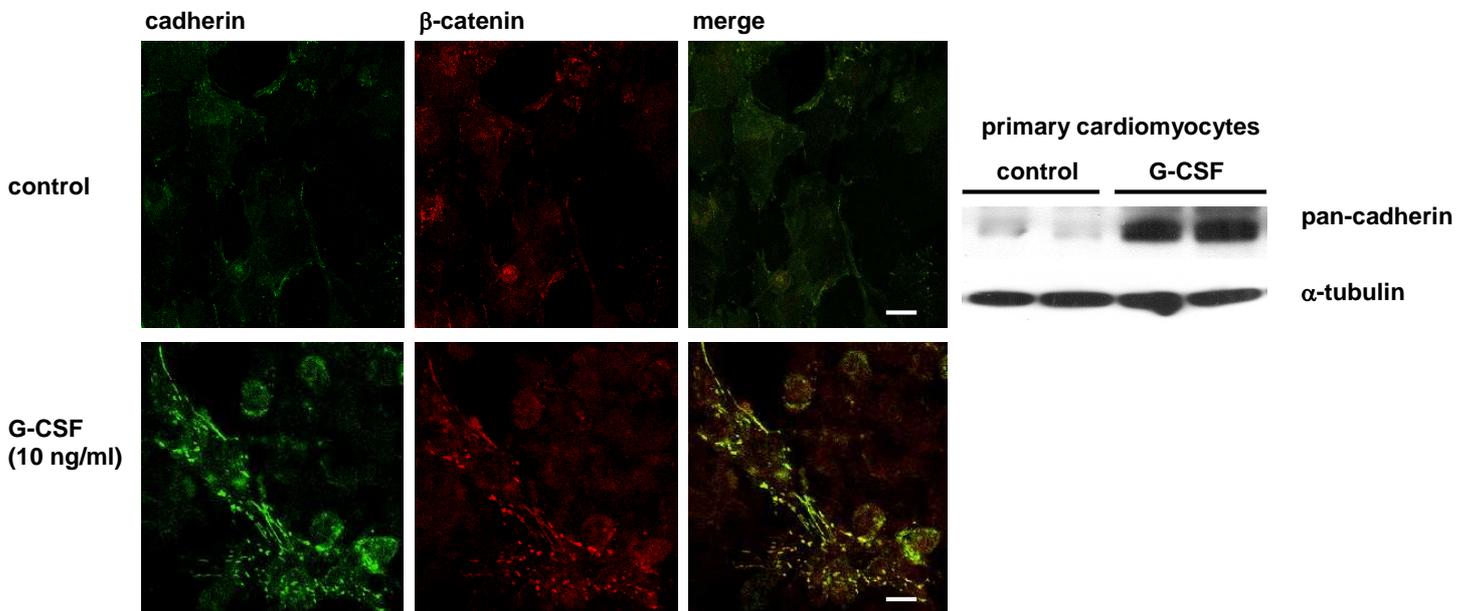


Figure 6

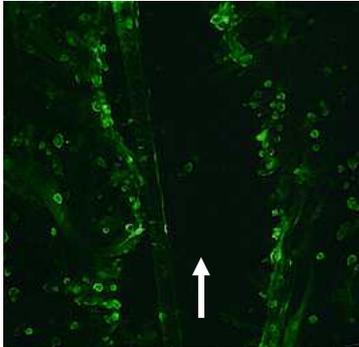


Kuwabara
Figure 7

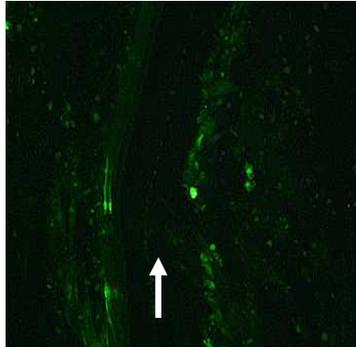
primary cardiomyocytes

A

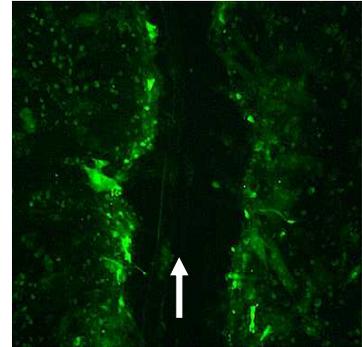
control (normoxia)



hypoxia

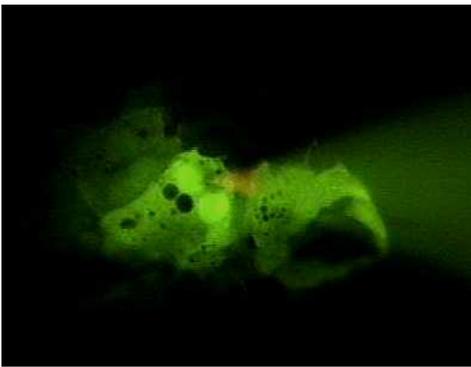


10 ng/ml G-CSF
+ hypoxia



B

normoxia



chemical hypoxia with CoCl_2



10 ng/ml G-CSF
+chemical hypoxia with CoCl_2

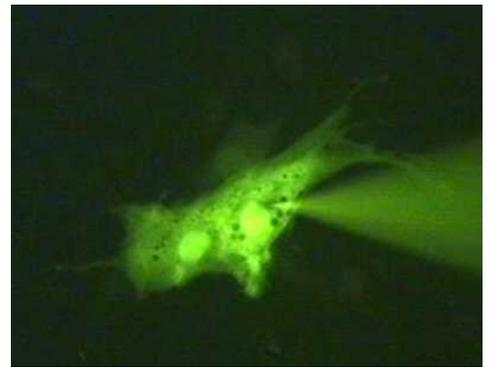
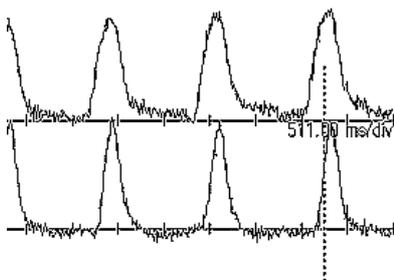
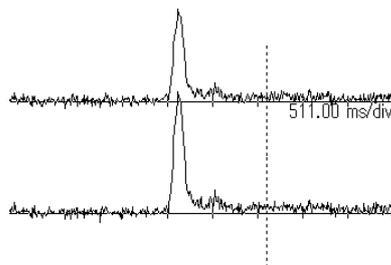


Figure 8

normoxia



hypoxia



10 ng/ml G-CSF
+ hypoxia

