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Clenbuterol Plus Granulocyte Colony-Stimulating Factor Regulates Stem/Progenitor Cell Mobilization and Exerts Beneficial Effect by Increasing Neovascularization in Rats With Heart Failure

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Abstract

Background—Treatment of beta₂-adrenergic receptor agonists with myeloid cytokines, such as granulocyte colony-stimulating factor (G-CSF) has been reported to enhance stem/progenitor cell mobilization and proliferation in ischemic myocardium. However, whether the combination therapy of G-CSF and clenbuterol (Clen) contributes to improved left ventricular (LV) function remains uncertain. We investigated whether this combination therapy induced bone marrow–derived stem/progenitor cell mobilization, neovascularization, and altered LV function after acute myocardial infarction (MI).

Methods and Results—Following MI, rats were treated with single Clen, high-dose Clen, and G-CSF + Clen. We evaluated LV function and remodeling with the use of echocardiography in addition to hemodynamics 3 weeks after MI. Treatment with G-CSF + Clen increased ($P < .05$), compared with no treatment, LV ejection fraction $46 \pm 3\%$ vs $34 \pm 2\%$, LV dP/dt $5,789 \pm 394$ mm Hg vs $4,503 \pm 283$ mm Hg, and the percentage of circulating CD34+ cells, appearing to correlate with improvements in LV function.

Conclusions—Combination therapy improved LV function 3 weeks after MI, suggesting that G-CSF + Clen might augment stem/progenitor cell migration, contributing to tissue healing. These data raise the possibility that enhancing endogenous bone marrow–derived stem/progenitor cell mobilization may be a new treatment for ischemic heart failure after MI.

Keywords

Neovascularization; beta₂-adrenergic receptor agonist

The epidemic of chronic heart failure (CHF) is growing, and advances in the treatment of coronary artery disease have led to an increased population of survivors with acute ischemic syndromes and LV dysfunction. Almost two-thirds of systolic heart failure is caused by coronary artery disease and at least one-half of patients with heart failure have a depressed ejection fraction (EF).¹ Approximately 15 million people worldwide and 5 million in the USA are currently diagnosed with CHF; an estimated 400,000 new cases per year will

present in the USA alone. CHF is the leading cause of hospitalization in USA patients >65 years old and constitutes the USA's largest diagnosis-related group with an annual in-patient volume of >600,000.^{2,3}

Earlier work suggests that granulocyte colony-stimulating factor (G-CSF) may play an important role in cardiac repair by inducing the generation of new cardiac tissue through either mobilizing bone marrow-derived stem cells, direct effects on cardiomyocytes,⁴⁻⁸ or the release of proangiogenic mediators.⁹⁻¹² However, recent experimental and clinical studies show no beneficial effect of stem cell mobilization by single G-CSF administration on ventricular recovery after acute myocardial infarction (MI).¹³⁻¹⁶ Clenbuterol (Clen), a β_2 -adrenergic receptor (β_2 -AR) agonist has been shown to be therapeutically beneficial when combined with left ventricular (LV) unloading in patients with severe CHF.¹⁷ Clen has been proposed to prevent unloading-induced atrophy and consequential myocardial dysfunction together with other agents.¹⁸ In addition, Clen has been shown to reduce myocardial cell death and lessen LV remodeling in the rat model.¹⁹

Recent data suggest that pharmacologic intervention with β_2 -AR agonists could be used therapeutically to mobilize stem cells which are stimulated by G-CSF directly or indirectly.^{20,21} Additionally, expression of β_2 -AR has been detected in enriched populations of mouse myeloid progenitors,²² indicating that interrelations between neuronal and stem/progenitor signaling mechanisms are possible. Therefore, we tested the hypothesis that the combination of G-CSF and Clen could have beneficial effects on LV function and remodeling in ischemic heart failure through bone marrow-derived stem/progenitor cell mobilization and neovascularization.

Methods

Heart Failure

All experimental animal protocols described herein were approved by the Animal Use Committees of the Southern Arizona VA Health Care System and the University of Arizona, in compliance with the Guide for the Care and Use of Laboratory Animals (www.nap.edu/catalog/5140.html). Heart failure is created in rats with the use of standard techniques developed in our laboratory.²³ In brief, Sprague-Dawley rats were infarcted by permanent ligation placement around the proximal left coronary artery. Rats undergoing this procedure develop large MIs as confirmed by LV end-diastolic pressure >16 mm Hg and the presence of a transmural scar. To ensure a consistent degree of myocardial injury, only rats with EF <40% were enrolled in the study as CHF or CHF + treatment. The treatment groups for this study were: 1) CHF + low dose ($1.6 \text{ mg kg}^{-1} \text{ d}^{-1}$) Clen; 2) CHF + high dose ($10 \text{ mg kg}^{-1} \text{ d}^{-1}$) Clen; and 3) CHF + $100 \text{ } \mu\text{g kg}^{-1} \text{ d}^{-1}$ G-CSF for 5 days + $1.6 \text{ mg kg}^{-1} \text{ d}^{-1}$ Clen for 2 weeks (G-CSF + Clen). In addition, both sham and CHF control animals were generated for comparison. Sham-operated animals underwent thorotomy and suture placement without ligation and CHF rats received saline injections at time points mirroring the treatment groups.

Echocardiography

We performed closed chest transthoracic echocardiography with the use of a Vingmed Vivid 7 system echo machine (GE Ultra-sound) with Echopac (GE Ultrasound) programming software and a 10-MHz multiplane transducer as previously described.²³⁻²⁵

Hemodynamic Measurements in Vivo

With the use of standard techniques developed in our laboratory, rats were anesthetized with inactin (100 mg/kg intraperitoneal injection) and a 2-F solid state micromanometer-tipped

catheter with 2 pressure sensors (Millar Instruments, Houston, Texas) was inserted via the right femoral artery, with one sensor located in the left ventricle and another in the ascending aorta.²⁶

LV Pressure-Volume Relationships

The LV pressure-volume relationship was measured as outlined in our previous work.^{23,24} In brief, hearts were arrested with potassium chloride and a catheter inserted into the LV via the aortic root. One end of the double-lumen LV catheter was connected to a volume infusion pump (Harvard Apparatus) and the other end to a pressure transducer zeroed at the level of the heart. The LV was filled (1.0 mL/min) to 60–100 mm Hg and unfilled while pressure was recorded onto a physiologic recorder (Gould). Ischemia time was limited to 10 minutes. Volume infused is a function of filling rate.

Flow Cytometry

Blood was drawn from the tail vein catheter, anticoagulated with sodium citrate (Sigma), and diluted with Pharmingen Staining Buffer (BD Biosciences). Diluted whole blood was aliquotted into 2 samples. Both samples were incubated for 25 minutes with phycoerythrin-Cy5-conjugated anti-CD45 (BD Pharmingen), a panleukocyte marker. One sample was treated with an IgG isotype control and the second sample with fluorescein isothiocyanate-conjugated anti-CD34 (Santa Cruz Biotechnology). Blood samples were fixed with cold 1% paraformaldehyde before analysis.²⁷ A FACS Caliber flow cytometer (Becton Dickinson) was used to acquire the samples. A total of 100,000 events were acquired and the monocyte population gated to identify the CD34-positive cells. Cell Quest Pro software was used for analysis, and the total fluorescence intensity (TFI) was calculated as the product of the values given for the “percent gated” and “geometric mean” of the M2 region.²⁸

Reverse-Transcription PCR

Reverse-transcription polymerase chain reaction (PCR) was performed as described previously.²⁹ Total RNA was harvested from neonatal rat cardiac myocytes 24, 48, and 72 hours after G-CSF + Clen treatment or from rat cardiac tissues with the use of Trizol (Invitrogen) and quantified with the use of Nanodrop (Thermo Scientific). cDNA was synthesized from 1 mg total RNA with the use of Superscript II Reverse Transcriptase (Invitrogen). Ten microliters of template cDNA (diluted 1:10) was used in a 20-μL PCR reaction with 26–29 cycles. PCR reagents were from the ExTaq kit (Takara Bio). Primers used included: rat stromal-derived factor 1 (SDF-1; forward 5'-TTGCCAGCACAAAGACACTCC-3', reverse 5'-CTCCAAAGCAAACCGAATACAG-3', to amplify a 226-bp product^{30,31}); rat insulin-like growth factor 1 (IGF-1; forward 5'-CAGACGGGCATTGTGGAT-3', reverse 5'-AGTCTTGGGCATGTCTAGTGTG-3', to amplify a 135-bp product³²); rat b₂-AR (forward 5'-GAGCCACACGGAATGACA-3', reverse 5'-CCAGGACGATAACCGACATGA-3', to amplify a 133-bp product³³); and rat GAPDH (forward 5'-CCAGTATGATTCTACCCACGGC-3', reverse 5'-CGGAGATGATGACCCTTTTGGC-3', to amplify a 227-bp product).

HistopathologydMicrovessel Density

Hearts were formalin fixed (10%), the right ventricle removed, and the heart cut in half along the midline of the left ventricle before paraffin embedding. Three-micrometer-thick sections were cut for staining and were produced within 15 μm of the sectioning plane for all samples. HIER antigen retrieval was performed in citrate-based Diva buffer (pH 6.2) for 1 minute at 125°C with the use of a “Decloaker” pressure cooker (Biocare Medical, Concord, California). We used single immunohistochemical staining for antirabbit factor VIII (Dako) (1:1,000) to perform histologic analysis of microvessel density, as previously

described.²³ We defined vessel density by light microscopy at $\times 40$ magnification. The number of cross-section vessels per field was counted by 2 people blinded to treatment, and the average measurements from 6 different fields was recorded for each value. Knowing the area of the optical field, data were reported as number of vessels/ μm^2 . Positive factor VIII microvessel staining was quantified within the epicardium, myocardium, and endocardium. These areas were defined as $65 \pm 10 \mu\text{m}$ from the outer surface of the heart, $65 \pm 10 \mu\text{m}$ midline of the LV wall, and $65 \pm 10 \mu\text{m}$ from the lumen of the ventricle, respectively.

Statistical Analysis

Data were expressed as mean $\pm$ SE. For the physiologic and echocardiographic measurements, the Student *t* test was used for single comparison of sham versus other study groups. Interactions were tested with the use of 2-way analysis of variance, and intergroup differences were evaluated with the use of the Student-Newman-Keuls test for statistical significance ($P < .05$). Pressure-volume relationships were evaluated with the use of multiple linear and polynomial regression analysis. The correlation of statistical difference was based on the Durbin-Watson statistic, F-statistic, *P* value, and variance coefficients.

Results

Hemodynamic Data

Three weeks after MI, rats had developed heart failure resulting in a decrease in LV systolic pressure, mean arterial pressure, prolonged tau, and peak developed pressure with increases in LV end-diastolic pressure (Table 1). Low-dose Clen increased peak developed pressure whereas high-dose Clen increased LV systolic pressure and mean arterial pressure. The combination therapy of Clen and G-CSF had the most beneficial hemodynamic effect as observed by increases in $+\text{LV dP/dt}$ and peak developed pressure and decreases in LV end-diastolic pressure and $-\text{LV dP/dt}$. There was no observable change in heart rate with any treatment, except for a decrease with Clen alone compared with sham.

LV Function Echocardiogram

After MI, the onset of CHF is characterized by progressive dilatation of the LV with a decrease in EF. Treatment of rats in CHF with G-CSF + Clen suggests a trend toward an increase in EF and decrease in LV end-diastolic volume (Table 2). These data in conjunction with the hemodynamic outcomes show that combination therapy of G-CSF + Clen improved LV function and partially reversed LV remodeling in rats with CHF.

Diastolic Pressure-Volume Relation

The diastolic pressure-volume relationships showed dilation of the left ventricle with the onset of CHF; administration of Clen and G-CSF in combination attenuated this dilation with a shift of the pressure curve to the left ($P < .05$) toward the pressure axis (Fig. 1).

Flow Cytometry

To determine the underlying mechanism of bone marrow stem/progenitor cell mobilization, we performed flow cytometry analysis of peripheral blood at days 3, 7, and 21 after MI (Table 3). We found that treatment with G-CSF + Clen increased ($P < .05$) CD34⁺ cells in the peripheral blood at day 3 ($53.38 \pm 1.46\%$ vs $62.33 \pm 2.47\%$) and day 7 (59.10 ± 5.7 vs $67.36 \pm 1.89\%$), but not day 21, correlating with improvements in LV function and hemodynamics. These results indicate that the combination of G-CSF + Clen augments stem/progenitor cell mobilization from bone marrow to peripheral blood and could potentially contribute to tissue healing in rats with ischemic heart failure.

Histopathology and Microvessel Density

We performed histopathology to determine if stem/progenitor cells mobilized from the bone marrow potentially contributed to tissue healing in rats with ischemic heart failure (Fig. 2). Epicardial, myocardial, and endocardial tissue was analyzed for microvessel density (vessels/ μm^2). The most dramatic improvements ($P < .05$) in microvessel density were found to be in epicardial tissue (Table 4). We also investigated the expression of SDF-1 and IGF-1 mRNA on cardiomyocyte and cardiac tissue treated with G-CSF and Clen at days 1 and 3. There was no up-regulation of either SDF-1 or IGF-1 mRNA on both cardiomyocyte and cardiac tissue at days 1 and 3 (data not shown).

Discussion

The results of the present study show that combination therapy with G-CSF + Clen: 1) stimulates bone marrow-derived stem/progenitor cell mobilization; 2) augments neovascularization; and 3) improves LV function with a trend to attenuate LV remodeling in rats with ischemic heart failure. Our data are consistent with earlier reports in which G-CSF + Clen combination therapy had been shown to improve LV systolic function and decrease LV chamber volume compared with Clen alone.³⁴ To our knowledge, this is the first report demonstrating favorable effects of G-CSF + Clen combination therapy on LV function in rats with ischemic heart failure. Although the contributing mechanisms remain unclear, we found that G-CSF + Clen combination therapy increases stem/progenitor cell mobilization, resulting in an increased number of circulating CD34+ cells early after MI and decreasing by 3 weeks. In addition, microvessel formation, as observed through positive factor VIII staining increased ($P < .05$), specifically within the endocardial infarct region. Although the main driver of microvessel formation appeared to correlate with Clen administration, functional improvements were not observed except as a dual therapy with G-CSF. Furthermore, the observed circulating endothelial precursor cell mobilization after MI correlates with data generated from clinical studies, thus suggesting that the combined pharmacologic approach outlined here is potentially clinically applicable.³⁵

To identify the potential regulatory mechanism of stem/progenitor cell recruitment, we investigated cytokine mRNA expression levels of SDF-1 and IGF-1, which have been shown to have a positive correlation with SDF-1.³⁶ SDF-1 has been shown to enhance in the recruitment of stem/progenitor cells to ischemic tissue.³⁷ However, contrary to our expectation, no up-regulation of either SDF-1 or IGF-1 mRNA were observed in tissue culture cardiomyocyte populations or cardiac tissue with or without G-CSF + Clen treatment after 1 and 3 days. Although earlier investigators have reported up-regulated mRNA expression of SDF-1 within 4 days after MI,³¹ other groups showed expression during MI, peaking at 1 week, and remaining through 6 weeks.³⁸ It was therefore predicted that SDF-1 mRNA up-regulation would be observed at a later time point, at least after 1 week after MI. Additionally, we expected IGF-1 mRNA up-regulation after MI owing to the correlation between SDF-1 and IGF-1 mRNA levels shown in previous clinical evaluation.³⁶ However, earlier clinical evaluation occurring at the time of LV assist device implantation revealed elevated IGF-1 mRNA levels, suggesting delayed and therefore not acute expression of IGF-1 mRNA. Taking together the work highlighted in peripheral studies and the results reported in the present study, it can be assumed that evaluation of both SDF-1 and IGF-1 mRNA levels 1 to 3 days after MI precede physiologic mRNA expression and should be evaluated at later time points.

Interestingly, a recent report has shown that β_2 -AR expression on immature CD34+ cells is up-regulated in vitro after exposure to G-CSF.³⁹ Therefore, we hypothesized that in vivo administration of G-CSF would up-regulate cardiomyocyte β_2 -AR mRNA expression, promoting β_2 -AR agonists effects. However the findings in the present study showed that β_2 -

AR mRNA expression was not up-regulated on cardiomyocyte or myocardial tissue 24, 48, or 72 hours after MI when treated with a β_2 -AR agonist (data not shown). The mechanism of augmentation of stem/progenitor cell mobilization in the blood neovascularization appears to be from the synergistic effect of G-CSF + Clen but may not be through β_2 -AR up-regulation.

Furthermore, enhanced hematopoietic stem progenitor cell migration has been reported with β_2 -AR agonist (Clen) and G-CSF administration when compared with G-CSF alone in both control and norepinephrine-deficient mice.²⁰ That finding lends support to our hypothesis that the effect of β_2 -AR agonist (Clen) and not β -antagonist (β -blocker) in conjunction with G-CSF may facilitate bone marrow-derived stem/progenitor cell migration.

Clinically, β -AR agonists are thought to be contraindicated in patients with ischemic heart failure therapy owing to their positive chronotropic and inotropic effects. Studies examining the differential role of β -AR subtypes (β_1 -AR vs β_2 -AR) in regulating autonomic signaling have shown that β_2 -AR had no significant effect on heart rate whereas the β_1 -AR resulted in an overall heart rate depression.⁴⁰ Although our data show that high-dose Clen administration depressed heart rate, combination therapy (G-CSF + Clen) did not, suggesting that the combination therapy is not deleterious after MI in the rat. Finally, our data suggest that future investigations could explore potential pharmacologic stimulation of stem/progenitor cell migration as opposed to direct cell injections as cell-based therapy for heart failure after an acute MI.

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Fig. 1. Diastolic pressure-volume relation curves of sham (n = 8), untreated chronic heart failure (CHF; n = 5), and granulocyte colony-stimulating factor (G-CSF) + clenbuterol (Clen; n = 7). Data are mean $\pm$ SE.

Fig. 2.
Factor VIII stain of border region of the endocardial infarct zone. Brown coloring represents positive factor VIII stain. Red arrowheads denote microvasculature. Abbreviations as in Fig. 1.

Table 1

Hemodynamic Evaluation of Rats 3 Weeks after Myocardial Infarction With or Without Combination Therapy

	MAP (mm Hg)	tau (ms)	HR (beats/min)	LV SP (mm Hg)	+LV dP/dt (mm Hg/s)	-LV dP/dt (mm Hg/s)	LV EDP (mm Hg)	PDP (mm Hg)
Sham	111 ± 6	16 ± 1	285 ± 12	129 ± 7	7,290 ± 372	6,103 ± 432	6 ± 1	177 ± 7
CHF	85 ± 4 ^a	23 ± 1 ^a	252 ± 12	99 ± 4 ^a	4,503 ± 283 ^a	2,853 ± 232 ^a	22 ± 1 ^a	69 ± 13 ^a
Clen	95 ± 6	23 ± 2 ^a	257 ± 10	113 ± 6	5,161 ± 413 ^a	3,376 ± 361 ^a	24 ± 2 ^a	122 ± 8 ^{ab}
Clen-high	103 ± 5 ^b	24 ± 2 ^a	241 ± 10 ^a	117 ± 5 ^b	5,564 ± 434 ^a	3,207 ± 334 ^a	25 ± 3 ^a	125 ± 7 ^{ab}
G-CSF + Clen	98 ± 5	20 ± 2	256 ± 18	114 ± 6 ^b	5,789 ± 394 ^{ab}	3,766 ± 318 ^{ab}	20 ± 2 ^a	127 ± 4 ^{ab}

Data are mean ± SE. MAP, mean arterial pressure; HR, heart rate; LV, left ventricular; SP, systolic pressure; EDP, end-diastolic pressure; PDP, peak developed pressure; Sham (n = 10); CHF, untreated chronic heart failure (n = 10); Clen, Clenbuterol (n = 9); Clen-high, high-dose clenbuterol (n = 6); G-CSF + Clen, granulocyte colony-stimulating factor and clenbuterol (n = 9).

^a $P < .05$ vs Sham;

^b $P < .05$ vs CHF;

^c $P < .05$ vs G-CSF + Clen;

^d $P < .05$ vs Clen;

^e $P < .05$ vs Clen-high.

Table 2

Echocardiography Evaluation of Rats 3 Weeks after Myocardial Infarction With or Without Combination Therapy

	EF (%)	LVDd (mm)	ANTs (mm)	EDV (mm ³)	BWT (g)	LV Wt. (g)
Sham	66 ± 3	7.7 ± 0.3	1.5 ± 0.1	95 ± 7	308 ± 6	0.75 ± 0.04
CHF	34 ± 2 ^{ac}	9.4 ± 0.2 ^a	1.3 ± 0.1 ^a	176 ± 11 ^a	302 ± 5	0.65 ± 0.03 ^{ac}
Clen	35 ± 3 ^{ac}	9.6 ± 0.2 ^a	1.4 ± 0.1	188 ± 9 ^{ac}	307 ± 9	0.70 ± 0.04
Clen-high	37 ± 2 ^{ac}	9.2 ± 0.3 ^a	1.3 ± 0.1 ^a	176 ± 13 ^a	318 ± 7	0.77 ± 0.03 ^b
G-CSF + Clen	46 ± 3 ^a	8.8 ± 0.3 ^a	1.4 ± 0.1	157 ± 9 ^a	301 ± 10	0.77 ± 0.03 ^b

Data are mean ± SE. Sham (n = 12); CHF (n = 18); Clen (n = 12); Clen-high (n = 14); G-CSF + Clen (n = 18). EF, ejection fraction; LVDd, left ventricular dimension in diastole; ANTs, anterior wall thickness in systole; BWT, body weight; other abbreviations as in Table 1.

^a $P < .05$ vs Sham;

^b $P < .05$ vs CHF;

^c $P < .05$ vs G-CSF + Clen;

^d $P < .05$ vs Clen;

^e $P < .05$ vs Clen-high.

Table 3

Flow Cytometry Analysis of CD34+ Cell Mobilization in Peripheral Circulation at 3, 7, and 21 Days (%)

	Day 3	Day 7	Day 21
Sham	41.72 ± 1.15	39.64 ± 1.23	42.54 ± 1.65
CHF	53.38 ± 1.46 ^{ac}	59.10 ± 5.70 ^{ac}	56.94 ± 3.45 ^a
Clen	52.01 ± 1.63 ^{ac}	62.53 ± 1.10 ^{ac}	58.88 ± 1.47 ^a
Clen-high	52.15 ± 2.89 ^{ac}	62.01 ± 1.85 ^a	56.77 ± 1.72 ^a
G-CSF + Clen	62.33 ± 2.47 ^a	67.36 ± 1.89 ^a	64.06 ± 4.34 ^a

Data are mean ± SE. Sham (n = 9); CHF (n = 7); Clen (n = 9); Clen-high (n = 7); G-CSF + Clen (n = 10). Abbreviations as in Table 1.

^a $P < .05$ vs sham;

^b $P < .05$ vs CHF;

^c $P < .05$ vs G-CSF + Clen;

^d $P < .05$ vs Clen;

^e $P < .05$ vs Clen-high.

Table 4Microvessel Density Evaluation in Cardiac Tissue Following Clen and GCSF therapy (vessels/ μm^2)

	Sham	CHF	Clen	Clen-High	G-CSF + Clen
Epicardium	0.22 ± 0.02^{cbd}	0.020 ± 0.01^a	0.102 ± 0.02^{ab}	0.18 ± 0.25^b	0.022 ± 0.002^a
Myocardium	0.065 ± 0.02	0.032 ± 0.002^d	0.067 ± 0.01^{bcd}	0.04 ± 0.001^d	0.039 ± 0.002^d
Endocardium	0.115 ± 0.02^b	0.033 ± 0.003^{acde}	0.103 ± 0.02^b	0.12 ± 0.03^b	0.111 ± 0.001^b

Infarct microvessel density counts as reflected by positive factor VIII. Data are mean $\pm$ SE. Sham (n = 2); CHF (n = 3); Clen (n = 3); Clen-high (n = 3); G-CSF + Clen (n = 2). Abbreviations as in Table 1.

For each respective cardiac region:

^a $P < .05$ vs Sham;

^b $P < .05$ vs CHF;

^c $P < .05$ vs G-CSF + Clen;

^d $P < .05$ vs Clen;

^e $P < .05$ vs Clen-high.