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Research article

Therapeutic effects of paracrine factors secreted by human umbilical cord blood mononuclear cells in myocardial infarctions

Paracrine effects of cord blood cells

Robert J. Henning[1,*], Qing Zhu[2], and Xiao Wang[2]

[1] University of South Florida, 13201 Bruce B. Downs Blvd, Tampa, Florida
[2] The University of South Florida and First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China

* **Correspondence**: Email: roberthenningmd@gmail.com.

Abstract: Stem cell paracrine factors are beneficial in myocardial infarction (MI) treatment. However, specific stem cell factor effects on myocardial cytokines and their molecular pathways have not been precisely identified. We treated 44 rats with MIs with intramyocardial Isolyte or 4×10^6 human umbilical cord blood mononuclear cells (hUCBC) without immune suppression. We measured infarct sizes and myocardial cytokines. We then stressed isolated myocytes with H_2O_2 to simulate MIs in the absence and presence of paracrine factors from hypoxic hUCBC. We measured myocyte Akt protein kinase, which causes survival, and JNK and p38 protein kinases, which cause myocyte death. In Isolyte treated MIs, TNF-α increased from 6.1% to 51.3%, MCP increased from 5.6% to 39.8%, MIP increased from 8.1% to 25.9%, and IL-1 increased from 7.1% to 20.0%. In hUCBC treated MIs, inflammatory cytokines did not change and there was no hUCBC rejection. MI sizes averaged 30% in Isolyte treated rats and 10% in hUCBC treated rats ($p < 0.01$). Hypoxic hUCBC increased secretion of HGF by 338%, IGF by 200%, VEGF by 192%, PGF by 150%, IL-10 by 150%, and SCF and TIMP by 100% in comparison with non-stressed hUCBC ($p < 0.001$). H_2O_2 increased myocyte activation of JNK by 297% and p38 by 83% and increased myocyte necrosis by >60% (all $p < 0.01$ vs. normal myocytes). In myocytes treated with H_2O_2 and hUCBC paracrine factors, JNK and p38 activation decreased by ≥ 40%, while Akt activation and myocyte viability increased by >100% (all $p < 0.01$ vs. myocytes with H_2O_2) The Akt inhibitor API prevented hUCBC paracrine factor effects on myocytes. Addition of the JNK inhibitor SP600125 or p38 inhibitor SB203580 to myocytes with H_2O_2 plus hUCBC factors increased myocyte viability. We conclude that hUCBC secrete growth factors and anti-inflammatory cytokines that increase myocyte Akt activation and myocyte survival and decrease myocyte JNK, p38 and myocyte death in MIs.

Keywords: umbilical cord blood cells; stem cells; paracrine factors; growth factors; anti-inflammatory cytokines; inflammatory cytokines; Akt (Protein kinase B); c-Jun N-terminal kinase (JNK); p38; myocyte necrosis; myocardial infarction; cardiomyopathy

1. Introduction

Human embryonic stem cells are thought to be multipotent and capable of transdifferentiation [1–3]. In contrast, adult stem cells in bone marrow and in fat do not transdifferentiate and act by paracrine mechanisms [4–6]. We are investigating stem cells isolated from human umbilical cords and cord blood which are more primitive than adult stem cells but more mature than human embryonic stem cells. One milliliter of human umbilical cord blood, obtained after full-term infant delivery, contains approximately 8000 primitive hematopoietic progenitor cells and 10,000 multipotent stem/progenitor cells [7]. Mesenchymal stem cells are also present in umbilical cord blood in small numbers but are present in larger numbers from Wharton's Jelly in the umbilical cord [8]. Approximately 400,000 human cord blood units have been stored in blood banks and more than 20,000 allogeneic cord blood cell transplants have been performed worldwide for the treatment of patients with Fanconi anemia, aplastic anemia, beta-thalassemia, severe combined immune deficiency, X-linked lymphoproliferative syndrome, Hurler syndrome, Hunter's syndrome, Wiskott-Aldrich syndrome, acute lymphoid leukemia, acute and chronic myeloid leukemia, myelodysplastic syndrome, and neuroblastoma [9].

Hematopoietic and mesenchymal stem cells from umbilical cord blood are able to survive during severe hypoxia and free oxygen radical stress and maintain their potency due to peroxidases and catalases within these cells, which are able to catalyze free oxygen radicals and thereby significantly limit or prevent umbilical cell necrosis [10]. The fact that hUCBC are beneficial in the treatment of different human metabolic and hematological diseases and that cord blood cells can survive during severe hypoxia and free oxygen radical stress suggests to us that hUCBC can also be beneficial in limiting LV damage in myocardial infarctions.

In the present investigation, we wished to determine the *in-vivo* and *in-vitro* effects of human umbilical cord blood mononuclear cells (hUCBC), which contain 1–2% CD34 stem cells and approximately 1% CD 105 (SH2) stem cells, and whether these cells are capable of transdifferentiation to myocytes or act primarily by paracrine mechanisms. We therefore initially examined the effects of hUCBC in a rat model of myocardial infarction. We then investigated the effects of hUCBC conditioned media on isolated cardiomyocytes subjected to free oxygen radical stress with hydrogen peroxide (H_2O_2) in order to simulate the free radical stress that occurs in acute myocardial infarctions. The present investigation suggests that hUCBC act primarily by releasing growth factors and anti-inflammatory cytokines that limit or inhibit the myocardial expression of inflammatory cytokines and death promoting myocyte c-Jun N-terminal kinase (JNK) and p38 protein kinases. Moreover, the beneficial effects of hUCBC paracrine factors are significantly enhanced by free oxygen radical stress.

2. Materials and methods

2.1.Human umbilical cord blood mononuclear cells (hUCBC)

Human umbilical cord blood mononuclear cells or human cord blood were obtained from human cord cell blood banks (CordUse, CryoCell, Cambrex) and the specimens did not contain any identifying information of the original donors. The cells were stored at -196 ℃ in liquid nitrogen. The cord blood was rejected if the blood was positive for Human Immunodeficiency Virus (HIV), human T-lymphocytic virus, hepatitis, syphilis or cytomegalovirus. The mononuclear cell fraction of cord blood was obtained by Ficoll density gradient separation and cryopreserved at -196 ℃ in liquid nitrogen. The cryopreserved hUCBC were thawed at 37 ℃ and transferred into centrifuge tubes containing Isolyte S, pH 7.4 (Braun Medical). The cells were washed three times, centrifuged at 1500 rpm for 7 min, the supernatant discarded and the hUCBC viability determined by Trypan Blue dye exclusion technique. The hUCBC viability was greater than 90%. hUCBC have very large nuclei that occupy approximately 85% of the cell. This morphology readily distinguishes these cells from cardiac myocytes, which have eccentric nuclei in cells with cross striations, and polymorphonuclear leukocytes, which have polylobed nuclei. The hUCBC were not propagated in culture flasks. The hUCBC contained 1–2% $CD34^+$ stem cells and approximately 1% SH2 (CD105) positive stem cells as determined by fluorescent antibodies to CD34 and SH2 cells that were obtained from Invitrogen and Osiris, respectively, and fluorescent activated cell sorting cytometry (Becton Dickinson) in our facility. The remaining mononuclear cells consisted primarily of hematopoietic precursor cells.

2.2. Myocardial infarction protocol

All investigational protocols were reviewed and approved by our Institutional Review Board. Forty-four Sprague-Dawley rats (Harlan), weighing between 250 and 300 g, were anesthetized with 4% Isoflurane by inhalation, intubated, and placed on mechanical ventilator support with continuous 2.5–4% Isoflurane and oxygen anesthesia. The heart rate and arterial oxygen saturation were continuously monitored (Imed) during the surgery. A thoracotomy was performed through the left fifth intercostal space. The pericardium was opened. The left coronary artery was permanently ligated with 6–0 silk suture at the level of the left atrium in order to produce a myocardial infarction that was consistent in size and reproducible in rats weighing between 250 and 300 grams. Infarction of the anterior wall of the left ventricle was confirmed in each rat by the presence of discoloration of the anterior myocardial wall and decreased wall motion. Twenty-two rats were then given 0.3 mL Isolyte and 22 rats were given hUCBC, 4×10^6, in 0.3 mL Isolyte by direct intramyocardial (IM) injection. The chest in each rat was then closed in three layers with 3–0 Vicryl and 6–0 Prolene suture, and each rat was allowed to recover. Buprenorphine (0.05–0.5 mg/kg IM) was given for post-operative analgesia every 6–8 hours for 24–48 hours. Immunosuppressive therapy was not given to any rat.

2.3. Echocardiography

Echocardiograms were obtained on controls (n = 5), Isolyte-treated infarcted rats (n = 5) and hUCBC treated infarcted rats (n = 5) prior to infarction and at 2 weeks after infarction. This technique is accurate and reproducible in normal rats and rats with myocardial infarctions [11]. A Siemens/Acuson echocardiographic system (Siemens AG., Erlangen, Germany) with a 15-MHz transducer with a resolution of < 0.5 mm was used. Each rat was lightly anesthetized with isoflurane 2% and 2D and M-mode echocardiograms were obtained at the level of the left ventricular papillary muscles and the images digitized. Three to 5 cardiac cycles were measured and the values for each

measurement were averaged for each rat. An investigator and a sonographer who were unaware of the rat's treatment analyzed the images. The left ventricular end-diastolic diameter, which was measured at the onset of the electrocardiographic QRS complex, was measured from the parasternal long-axis or apical views. The left ventricular end-systolic diameter was measured after aortic valve closure or measured from the frame in which the left ventricular chamber dimension was the smallest. Left ventricular fractional shortening was determined from LV End-Diastolic diameter (LVED) minus Left Ventricular End-Systolic diameter (LVES) divided by LVED × 100.

2.4. Tetrazolium staining for infarct sizing

A separate group of Isolyte (n = 6) and hUCBC, 4×10^6, treated hearts (n = 6) at 72 hours after infarction were cut from apex to base in four separate 2.5 mm slices. Each slice was placed in 1% triphenyltetrazolium pH 7.4 and 37 ℃ for 40 minutes. The slices were rinsed with cold saline and fixed in 10% formalin. Each heart slice was photographed with a digital camera and the images transferred to a computer workstation. The area of infarction and the area of the normal ventricles were then determined by Image Pro software (Media Cybernetics). The areas for each slice were then summed to determine the total infarct area and the total area of the right and left ventricle for each heart. Infarct size was then expressed as the infarct area divided by the total left plus right ventricular muscle area. Tetrazolium does not stain autolytic myocardium or hUCBC and has a diagnostic efficiency of 88% for infarcted myocardium [12]. All measurements were determined by two separate investigators and the results averaged.

2.5. Myocardial histopathology

A pathologist, who was unaware of the treatment for each rat, examined heart tissue sections from randomly selected rats from each group. Myocardial tissue was stained with hematoxylin and eosin. In addition, myocardial tissue was stained with antibodies to human nuclear antigen (Abcam). The tissue slides were examined with a Zeiss microscope and an Olympus fluorescent microscope. Selected tissue sections were digitized and examined on a workstation.

2.6. Myocardial cytokine determination

Hearts from the rats at 0, 6, 12, and 24 hours (n = 3–4 at each time) after coronary artery ligation and the creation of myocardial infarction were flash frozen in liquid nitrogen and then stored at -86 ℃. The hearts were then thawed at 25 ℃ and the ventricles dissected free from the heart. The ventricular tissue was placed in 750 μL of lysis buffer that contained 20 mM Tris, pH 7.5, 0.3 M NaCl, 2% sodium deoxycholate, 2% TX-100, plus a Protease Inhibitor Cocktail (Roche) and homogenized until there was no visible tissue. The heart homogenate was placed on a rocker plate for 2 hours at 4 ℃ and then centrifuged at 12,000 RPM for 30 minutes at 4 ℃.

The protein concentration of the supernatant was then determined by the Bradford Assay and bovine serum albumin was used as a standard. Fifty μg of protein from each ventricular supernatant was added to 2 mL of blocking buffer (RayBiotech) and each solution was placed on a separate cytokine array membrane (RayBiotech) in a plastic tray and incubated for 3 hours at room temperature on a rocker plate. The solution was then aspirated and each membrane incubated for 12 hours at 4 ℃ with a mixture of

biotinylated cytokine primary antibodies, based on our preliminary studies, specific for tumor necrosis factor-alpha (TNF-α), monocyte chemoattractant protein (MCP), macrophage inflammatory protein (MIP), interferon-gamma (IFN-γ), interleukin (IL) IL-1 (RayBiotech) on a rocker plate according to the manufacturer's instructions. The membranes were rinsed and HRP conjugated secondary antibodies were added to each membrane. The membranes were incubated at room temperature for 2 hours, subjected to an enhanced chemiluminescence detection kit (Amersham) for 60 seconds, and exposed on radiographic film (Amersham) for 90 seconds. The blots on the radiographic film were scanned and the protein densities determined with ImagePro image analysis software (Media Cybernetics). All measurements were performed in duplicate and the results averaged for each measurement. The results for each cytokine were then compared with controls. Mean ± SEM values were then determined.

2.7. Protein microarrays

Protein microarray experiments were performed on media from hUCBC in DMEM or from media from hUCBC with DMEM after 12 hours of hypoxia. Two milliliters of media from each experiment was added to 2 mL of blocking buffer (RayBiotech). The solution from each experiment was placed on a separate protein array membrane (RayBiotech) and incubated for 2 hours at room temperature on a rocker plate. The solution was then aspirated, and each membrane was incubated for 12 hours at 40 °C with a customized mixture of biotinylated primary antibodies, based on our preliminary experiments, which were specific for hepatocyte growth factor (HGF), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), placental growth factor (PGF), angiogenin, and interleukin 10 (IL-10) on a rocker plate [13]. The membranes were rinsed, and horseradish peroxidase-conjugated streptavidin was added to each membrane. The membranes were incubated at room temperature for 2 hours, subjected to enhanced chemiluminescence detection and exposed on radiographic film (Amersham). The protein density expression level was determined with the use of ImagePro image analysis software (Media Cybernetics). All measurements were performed in duplicate, and the results averaged for each measurement. The variation in duplicate determinations was less than 10% [14]. The results for each growth factor or cytokine were then compared with positive controls present on each membrane. The measurements during hypoxia were then expressed as a percentage of the measurements of the hUCBC in DMEM without hypoxia. The protein microarrays used can detect as little as 10 pg/mL of growth factor or cytokine protein [14].

2.8. Myocyte cell cultures

Rat cardiac myocytes (H9c2) were obtained from the American Type Culture Collection (CRL-1446). H9c2 myocytes have morphological characteristics similar to those of embryonic cardiac myocytes but have hormonal, enzymatic and electrical signal pathways similar or identical to adult cardiac myocytes [15,16]. The cardiac myocytes were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) with 5% fetal bovine serum (Fisher) and penicillin-streptomycin (Invitrogen) and grown to approximately 80% confluence in T25 polyethylene tissue culture flasks (BD Bioscience).

2.9. hUCBC-conditioned medium

Six million hUCBC were plated with DMEM with 10% fetal bovine serum in T25 flasks (Fisher

Scientific) in 10 experiments. The following day, the hUCBC cultures were serum starved with basal culture media without fetal bovine serum and subjected to hypoxia (1% O_2, 94% N_2, and 5% CO_2). After 12 hours, the medium without cells was collected and labeled as hUCBC-conditioned medium. The hUCBC conditioned media was filtered with a 0.22 μm cellulose syringe filter (EMD Millipore) and subjected to protein microarray analysis. The stressed hUCBC conditioned media was used to treat cardiomyocytes that were subjected to H_2O_2 in order to determine the effects on cardiomyocyte viability.

2.10. Myocyte and hUCBC protocols

Cardiac myocytes or cardiac myocytes plus hUCBC conditioned media in culture were serum starved for 24 hours and were then subjected to 100 mmol/L H_2O_2 for 12 hours. Cell viability assays and Western blots were then performed on each group.

2.11. Cell viability assay

Trypan blue assays were performed on cardiac myocytes from serum-free cultures after 12 hours of treatment with either 100 mmol/L H_2O_2 or with 100 mmol/L H_2O_2 plus hUCBC conditioned media. Cells that did not stain with Trypan blue were counted as viable cells. Conversely, cells that stained with Trypan blue were counted as non-viable cells. The cell counts were expressed as percentages of the control cells that were not treated with H_2O_2.

2.12. AKT, JNK, and p38 inhibitor studies

Cardiomyocytes in cultures or myocyte plus hUCBC conditioned media were pretreated for 30 min with or without the sensitive and specific Akt inhibitor API (10 mmol/L) [17], the JNK inhibitor SP600125 (10 μmol/L, Fisher) [18,19] or the p38 inhibitor SB203580 (10–40 μmol/L, Fisher) [20,21]. Thereafter, H_2O_2 was added to the cultures for 12 hours.

2.13. Western blot analysis

Cardiac myocytes were lysed with radioimmunoprecipitation assay buffer (Cell Signaling) containing protease and phosphatase inhibitors (Pierce Chemical). Protein determinations were performed with the use of a bicinchoninic acid protein kit [Pierce Chemical] with bovine serum albumin as the standard. Equal amounts (50 μg) of protein were loaded and separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were then blocked for 1 hour in Tris-buffered saline with Tween-20 buffer containing 5% non-fat milk. Immunoblotting was performed by incubating the membranes with p-JNK (EMD Millipore), p-p38 (Cell Signaling) or p-Akt and Akt (Cell Signaling) primary monoclonal antibodies, in 5% bovine serum albumin, overnight at 4 ℃ and then with secondary antibodies conjugated with horseradish peroxidase overnight at 4 ℃. After washing, the protein bands were detected by means of Supersignal West Pico chemiluminescence kits (Thermo Scientific). The results were quantified through the use of Image J software (National Institutes of Health).

2.14. Data analysis

All values are expressed as mean standard ± error of the mean. The significance of differences between values was assessed by means of Student's t-test. When multiple comparisons among groups were performed, repeated-measures analyses of variance were performed. Thereafter, the Bonferroni modification of the t-test was used for planned comparisons and Tukey's procedure was used for post hoc comparisons. Statistical significance was assigned at a value of $p \leq 0.05$.

3. Results

3.1. Myocardial cytokines in infarcted myocardium

Inflammatory cytokines in rats with myocardial infarctions were determined immediately after coronary artery ligation and the creation of the myocardial infarction (0 hours), then at 6, 12, and 24 hours. The maximal increases in myocardial cytokines occurred at 12 hours after coronary ligation. Inflammatory tumor necrosis factor-α increased from 6.1 ± 0.9% to 51.3 ± 4.7%, monocyte chemoattractant protein increased from 5.6 ± 1.2% to 39.8 ± 2.1%, macrophage inflammatory protein increased from 8.1 ± 1.5% to 25.9 ± 1.4%, interleukin-1 increased from 7.1 ± 0.04% to 20.0 ± 1.2%, compared with normal controls. In contrast in rats with myocardial infarctions treated with intramyocardial hUCBC, the myocardial concentrations of these cytokines did not significantly change at 0, 6, 12, or 24 hours after coronary artery ligation and myocardial infarction. See Figure 1.

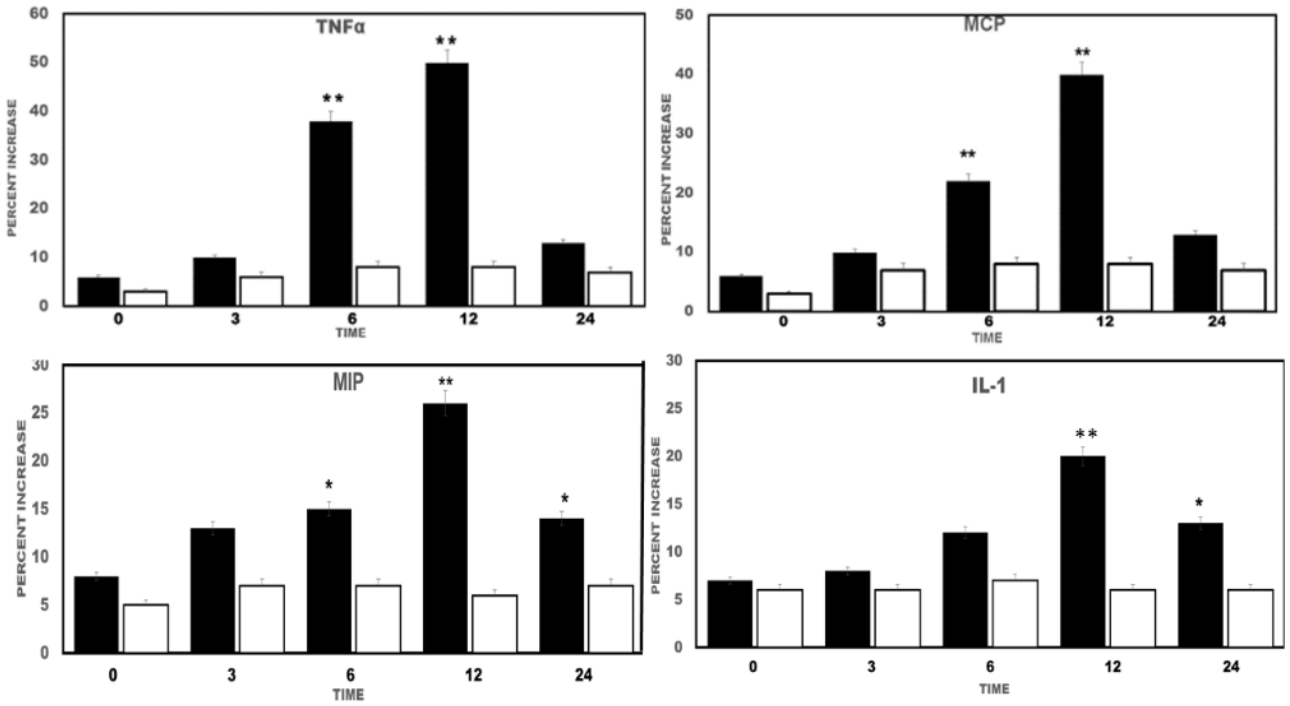

Figure 1. Inflammatory cytokines present in the myocardium after coronary artery ligation and the creation of myocardial infarction. TNFα-tumor necrosis factor alpha. MCP = Monocyte chemoattractant protein. MIP = Macrophage inflammatory protein. IL-1 = Interleukin-1. The Time scale is the time in hours after the coronary artery ligation and the creation of the acute myocardial infarction. White bars represent infarcted myocardium treated with hUCBC. Black bars represent infarcted myocardium treated with Isolyte. * = $p \leq 0.05$. ** = $p < 0.01$ in comparison with control measurements.

3.2. Echocardiographic changes with ischemic cardiomyopathies

Left ventricular fractional shortening averaged 54 ± 1% in the rats prior to any intervention. In the Isolyte treated rats with myocardial infarctions, the left ventricular fractional shortening significantly decreased after coronary ligation and averaged 24 ± 1.1% at 2 weeks ($p < 0.001$ in comparison with normal rat LV fractional shortening). The fractional shortening measurements in the hUCBC treated rats with myocardial infarctions were greater than the measurements in the rats in the Isolyte group. The fractional shortening measurements in the hUCBC Group averaged 34.1 ± 0.9 at 2 weeks post-infarction ($p < 0.01$ in comparison with the Isolyte treated rats).

3.3. Infarct size and histology

The infarct area and the total right and left ventricular areas were measured in the Isolyte treated rat hearts (N-6) and in the hUCBC treated rat hearts (N = 6) at 72 hours after infarction and the ratio Infarct Area/Total Right + Left Ventricular Area determined for the Isolyte and the hUCBC treated hearts. The infarct size ratio averaged 30 ± 8% in the Isolyte treated rats and 10 ± 3% in the hUCBC treated rats ($p < 0.01$). Representative horizontal sections of a tetrazolium stained Isolyte treated heart and a hUCBC treated heart are shown in Figure 2.

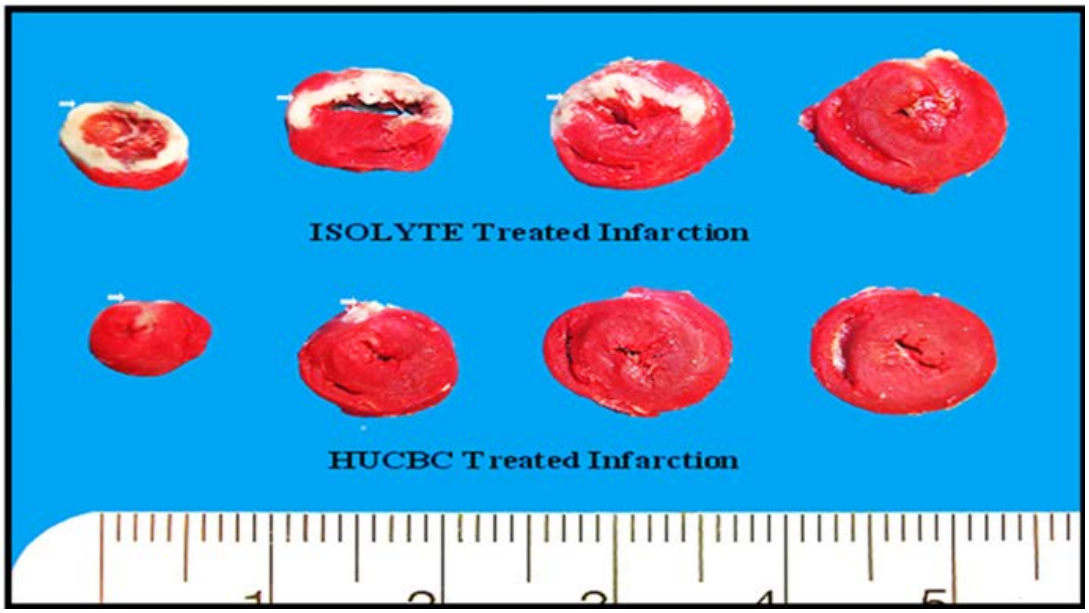

Figure 2. Representative horizontal sections of the right and left ventricles of a heart treated with Isolyte and a heart treated with hUCBC that were photographed with a digital camera with image size 3.2M (2048×1536). The sections have been stained with triphenyltetrazolium chloride which is enzymatically reduced to red 1, 3, 5-triphenyl formazan due to lactate dehydrogenases in the viable myocardium. Infarct necrosis areas are white (arrows) due to lack of lactate dehydrogenase enzymes. The hUCBC treated heart has significantly less ventricular necrosis than the Isolyte treated heart.

We did not detect in cardiac myocytes histological evidence of positive staining with antibodies to human nuclear antigen. In addition, we did not observe cardiac myocytes with early myofibrillar

organization or early Z band development that might indicate hUCBC transdifferentiation to cardiac myocytes.

3.4. Stress effects on hUCBC paracrine factors

In order to simulate changes that occur in the myocardium with infarctions, hUCBC in culture were serum starved for 24 hours and were then subjected to hypoxia (1% O_2, 94% N_2, and 5% CO_2) for 12 hours. Compared with hUCBC in normal DMEM, hUCBC stressed with hypoxia increased the secretion of hepatocyte growth factor by 348%, insulin-like growth factor by 199%, vascular endothelial cell growth factor by 195%, placental growth factor by 150%, interleukin-10 by 150% and stem cell factor and tissue inhibitory of metalloproteinase by 100% (all $p < 0.001$). See Figure 3.

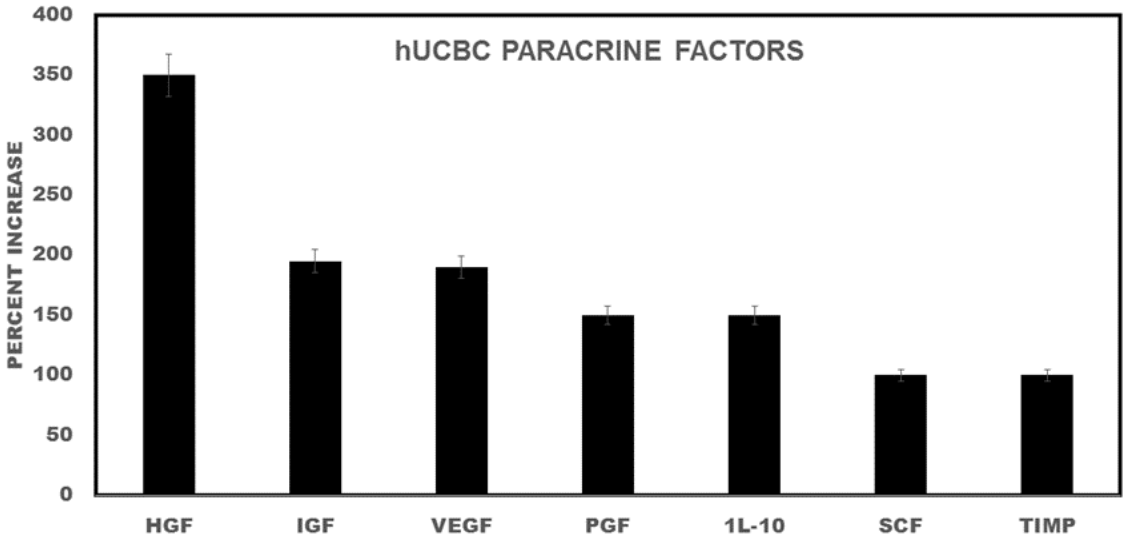

Figure 3. Percent increase in paracrine factors secreted by hUCBC that have been stressed with hypoxia (1% O_2, 94% N_2, and 5% CO_2) in comparison with non-stressed hUCBC in culture media. HGF = Hepatocyte growth factor. IGF = Insulin growth factor. VEGF = Vascular endothelial growth factor. PGF = Placental growth factor. IL-10= Interleukin 10. SCF = Stem Cell Factor. TIMP = Tissue inhibitor of metalloproteinase. All increases were significant at the $p < 0.001$ level in comparison with normal hUCBC media.

3.5. Hydrogen peroxide effects on cardiac myocytes

In cardiac myocytes without hUCBC media, serum starvation plus H_2O_2 increased myocyte JNK activation by 297% and p38 activation by 83% in comparison with control myocytes treated with only serum starvation ($p < 0.01$). See Figure 4A,B. The increase in JNK and p38 was associated with a decrease in myocyte viability and an increase in myocyte necrosis that was >60% ($p < 0.01$). See Figure 5. In contrast, hUCBC paracrine factors significantly increased Akt activation (p-AKT) in the myocytes treated with serum starvation and H_2O_2. See Figure 4C. The increase in Akt was associated with a decrease in JNK activation by 60% and p38 activation by 40% (all $p < 0.01$ in comparison with myocytes treated with H_2O_2). See Figure 4A,B. As a consequence, cardiomyocyte viability approached Control values. See Figure 5.

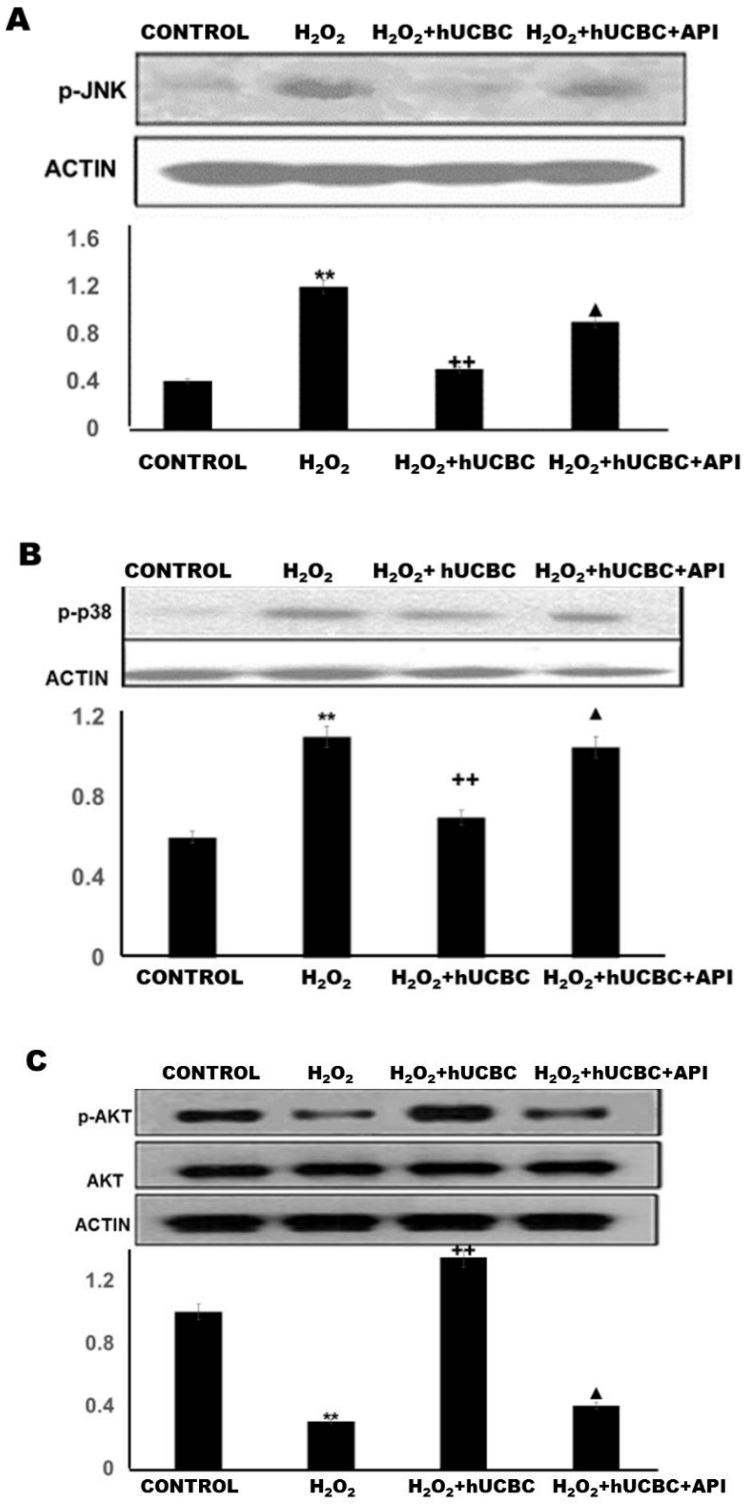

Figure 4. A–C: p-JNK, p-p38, AKT, and p-AKT expression in myocyte cultures with serum-starved media (CONTROLS), H_2O_2, or H_2O_2 plus hUCBC conditioned media with paracrine factors. Results are from four to six independent experiments. H_2O_2 significantly increased and hUCBC paracrine factors significantly decreased myocyte p-JNK and p-p38 expression. The Y-axis represents computer quantification of Western blot densities corrected for actin band densities.* = $p \leq 0.05$, ** = $p < 0.01$ in comparison with controls. ++ = $p < 0.01$ in comparison with H_2O_2. ▲ = $p < 0.01$ in comparison with H_2O_2 plus hUCBC paracrine factors.

3.6. Inhibition of AKT, JNK, and p38

The administration of the sensitive and selective Akt inhibitor API to myocytes treated with H_2O_2 plus hUCBC conditioned media nearly totally prevented the beneficial effects of hUCBC on Akt. See Figure 4C. Inhibition of Akt allowed H_2O_2 activation of JNK and p38 to levels seen in myocytes with serum starvation and H_2O_2 treatment alone ($p < 0.01$). See Figure 4A,B. In addition, API enhanced the H_2O_2 decrease of myocyte viability in comparison with H_2O_2 and hUCBC conditioned media ($p < 0.01$). See Figure 5.

The addition of JNK inhibitor SP600125 or p38 inhibitor SB203580 to myocytes plus H_2O_2 and hUCBC conditioned media consistently decreased the H_2O_2 reduction in myocyte viability and increased the beneficial effects of hUCBC paracrine factors. See Figure 5. In the presence of hUCBC paracrine factors, SP600152 and SB203580 inhibitors increased myocyte viability to almost control levels ($p = 0.05$ in comparison with H2O2 plus hUCBC). See Figure 5.

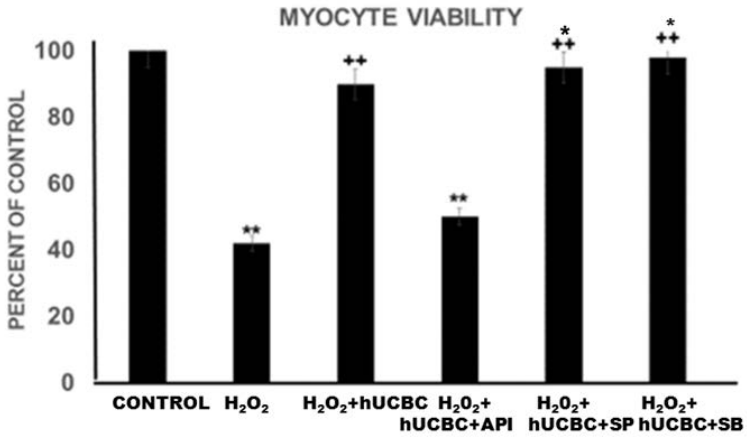

Figure 5. Viability experiments are shown with myocytes treated with H_2O_2, H_2O_2 plus hUCBC conditioned media, H_2O_2 plus hUCBC conditioned media plus API, and H_2O_2 plus hUCBC conditioned media plus JNK inhibitor SP600125 or the p38 inhibitor SB203580. SP600125 and SB203580 consistently increased the effect of hUCBC media on increasing myocyte viability during H_2O_2. The JNK inhibitor SP600125 increased myocyte viability to 96% and the p38 inhibitor SB203580 increased myocyte viability to 98% in comparison with H_2O_2 + hUCBC media. ** = $p < 0.01$ in comparison with controls. ++ = $p < 0.01$ in comparison with H_2O_2 and H_2O_2 plus hUCBC conditioned media plus API.

4. Discussion

The present investigation demonstrates that tumor necrosis factor-α, monocyte chemoattractant protein, macrophage inflammatory protein, and interleukin-1 are significantly increased in the ventricular myocardium with acute myocardial infarction. However, hUCBC paracrine factors, which are biologically active, significantly limit the expression of these inflammatory cytokines in the myocardium and increase cardiac myocyte survival by activating myocyte Akt and downregulating myocyte protein kinases JNK and p38. In this manner, hUCBC paracrine factors can significantly decrease infarct size and the reductions in ventricular contractility due to myocardial infarction.

4.1. Inflammatory cytokines in infarcted myocardium

Acute myocardial infarction generates free oxygen radicals in the myocardium which trigger a cytokine cascade that is initiated by Tumor Necrosis Factor-alpha (TNF-α) [22,23]. TNF-α, which is released from cardiomyocytes, macrophages, and mast cells, chemoattracts neutrophils which amplify the inflammation in myocardial infarction and promote tissue digestion and efferocytosis of dying myocytes and vascular endothelial cells. This cytokine has the unique ability to self-amplify through a positive feedback loop that involves the transcription factor NF-kB. Persistent over-expression of TNF in the myocardium decreases myocyte SERCA2a expression and diastolic calcium reuptake by the sarcoplasmic reticulum, and in this manner decreases cardiac contractility [24,25]. Conversely, when TNF expression is limited in the infarcted heart, myocardial cytokine expression is reduced and myocardial contractility is improved [26]. The present investigation demonstrates that hUCBC paracrine factors can produce as much as an 80% reduction in myocardial TNF-α expression. See Figure 1. This reduction in TNF-α was associated with a 73% reduction in infarct size and a 42% increase in left ventricular fractional shortening in comparison with rats with myocardial infarctions that were treated with Isolyte.

Myocardial monocyte chemotactic protein 1 (MCP), which in the present investigation increased six-fold in the Isolyte treated infarcted rat hearts, activates circulating macrophages, T lymphocytes, and mast cells and increases myofibroblast accumulation in the myocardium [27–29]. These cells release degradative lysosomal enzymes in the myocardium during acute infarction that contribute to the digestion of necrotic myocytes, ventricular fibrosis and ultimately ventricular remodeling. In addition, high concentrations of MCP with acute myocardial infarctions are associated with recurrent myocardial infarctions and increased patient mortality [28,30]. In contrast, MCP–knockout research animals exhibit decreased macrophage activation, delayed granulation tissue formation, and decreased left ventricular remodeling [29]. In the present experiments, limitation in the myocardial expression of MCP by as much as 80% by hUCBC paracrine factors was associated with a decrease in infarct size and LV remodeling after myocardial infarction. See Figure 1.

Macrophage inflammatory protein, which increased in the present experiments more than threefold in the Isolyte treated rats, activates neutrophils and induces the synthesis and release of IL-1, IL-6, and TNF-α from macrophages and fibroblasts [31]. In addition, MIP serum concentrations positively correlate with the size of myocardial infarctions and negatively correlate with LV ejection fractions [32]. In this regard, the myocardial MIP concentrations in the Isolyte treated rats in the present investigation were associated with infarct sizes that were threefold greater than the infarct sizes of the rats treated with hUCBC paracrine factors.

Interleukin-1 is released during myocardial infarction from cardiac myocytes, endothelial cells, and fibroblasts into myocardial tissue and helps to coordinate the inflammatory cellular response to myocardial infarction [33]. In addition, IL-1 stimulates the breakdown of extracellular matrix by metalloproteinases and ultimately contributes to the size of the infarction and LV ventricular remodeling. In the present experiments, IL-1 increased as much as fourfold in the myocardium of the rats treated with Isolyte but significant increases in IL-1 were prevented by hUCBC paracrine factors. See Figure 1. The importance of IL-1 in coronary artery disease and myocardial infarction is shown by the Cantos Study in which canakinumab, a human monoclonal antibody targeted against IL-1, significantly decreased the incidence of recurrent myocardial infarctions in patients with cardiovascular disease with chronic inflammation and increases in C-reactive protein [34].

Each of the cytokines present in the myocardium of the rats treated with Isolyte contributes to the inflammatory response to myocardial infarction and ultimately contributes to myocardial fibrosis and left ventricular remodeling. The persistence of these inflammatory cytokines in the myocardium, especially in combination, can lead to substantial left ventricular remodeling, ischemic cardiomyopathy, and increased risk for recurrent myocardial infarction and heart failure.

4.2. Stem cell growth factors and anti-inflammatory cytokines

In the present investigation, hUCBC stressed by hypoxia secreted hepatocyte growth factor, insulin-like growth factor, vascular endothelial cell growth factor, placental growth factor, interleukin-10, stem cell factor, and tissue inhibitor of metalloproteinase. See Figure 3. The concentration of these factors substantially exceeded the concentration of the same factors from non-stressed hUCBC. This suggests that hUCBC and hUCBC paracrine factors can survive in and respond to hostile environments such as myocardial infarctions and contribute to cardiac repair. In this regard, the growth factors hepatocyte growth factor, insulin-like growth factor, vascular endothelial cell growth factor, and placental growth factor have been shown to limit or prevent myocyte or vascular endothelial cell death [35–40]. Moreover, the hUCBC paracrine factors vascular endothelial growth factor, insulin growth factor, and hepatocyte growth factor can act additively or synergistically to stimulate myocardial neovascularization [41–43]. In addition, the paracrine factor IL-10 can decrease the release of free oxygen radicals from inflammatory cells, suppress neutrophil adhesion to vascular endothelium and neutrophil infiltration into infarcted myocardium, and downregulate TNF, IL-1, and metalloproteinases [44–47]. Consequently, hUCBC paracrine factors can repair the injured myocardium by acting on different inflammatory pathways in myocardial infarctions. Mesenchymal stem cell paracrine factors have also been reported to be beneficial in myocardial repair after myocardial infarction [5,6].

The critical paracrine factors that mediate the increase in myocyte viability and the decrease in infarct size in the present investigation are most likely a combination of all the biologically active factors described in the present report that work on different components of the myocardial infarction to limit inflammation and myocyte necrosis and enhance myocardial repair. The present investigation suggests that an important mechanism by which hUCBC paracrine factors limit myocardial injury and LV remodeling is by upregulating the expression and activation of the survival protein Akt in cardiomyocytes.

4.3. AKT activation

Akt is a serine/threonine-specific protein kinase that is necessary for cardiomyocyte survival and gene expression [48,49]. Akt contributes to myocyte survival by phosphorylation of the myocyte death proteins BAX and BAD, which prevents ischemia-induced mitochondria permeabilization and cytochrome C release into the cytoplasm of myocytes and endothelial cells [50]. Akt also contributes to myocyte survival by activating Forkhead transcription factors [51], increasing nitric oxide (NO) [52], regulating Ca^{2+} cycling [53,54], and contributing to cardiac stem cell survival [55]. In the present experiments, the hUCBC growth factor increases in myocyte Akt activity was associated with an increase in myocyte viability to levels that approached normal controls. See Figure 5. The fact that the increase in myocyte viability was due to Akt is supported by the fact that the increase in myocyte

viability was substantially inhibited by the sensitive and specific AKT inhibitor API. See Figures 4 and 5. In this regard, API inhibits Akt activation in myocytes by preventing Akt membrane localization and the full phosphorylation of Akt at threonine 309 and serine 474 [17]. However, API does not inhibit other important cell kinases such as Ras-dependent extracellular signal-regulated kinases (ERK1/2), mitogen-activated protein kinase, JNK, p38, protein kinase C, serine/threonine-protein kinase or protein kinase A [17]. Recently, stem cells have been shown to also secrete exosomes that contain microRNA-21 which can activate Akt and protect cells against H_2O_2-triggered cell death. In these studies, inhibition of the phosphoinositide-3 kinase-Akt pathway with LY294002 or inhibition of microRNA-21 prevented Akt activation and increased cell death [56].

4.4. JNK and p38 activation

Cardiomyocyte JNK is activated in myocardial infarctions by TNF, IL-1, and free oxygen radicals [57–59]. In the present investigation, the free oxygen radical generator hydrogen peroxide was used to significantly activate JNK [60]. Once activated, JNK can induce myocyte death by releasing cytochrome C from mitochondria and thereby disrupting ATP formation, activating the caspase cascade of cysteine proteases, activating the myocyte death proteins BAX and BAD, and inactivating the anti-death myocyte protein Bcl-2 [61–62]. Akt activation by hUCBC paracrine factors in the present investigations significantly limited or inhibited the activation of JNK and increased myocyte viability as shown in Figures 4 and 5. The increase in myocyte viability was due most probably to Akt induced increases in the activation of the myocyte Bcl-2 and the JNK interacting protein-1 [63,64]. Additional increases in the viability of myocytes treated with H_2O_2 to near control levels occurred in the present experiments with the application of the JNK inhibitor SP600125. See Figure 5. The fact that the viability of myocytes treated with H_2O_2 consistently increased to near control levels with SP699125 indicates that JNK is important in myocyte death.

p38-mitogen protein kinases are also strongly activated by inflammatory cytokines and chemical stressors such as H_2O_2 treatment. This protein kinase induces myocyte death by activating the myocyte death protein BAX, the Fas/Fas ligand cytotoxicity pathway, and the caspase cysteine protease cascade [65]. In contrast, p38-deficient cells, or myocytes treated with the p38 inhibitor SB203580, which was utilized in the present experiments, are resistant to cell death because of the downregulation of BAX and Fas/Fas Ligand cytotoxicity and enhanced activity of the ERK survival pathway [65]. In the present experiments, inhibition of p38 by SB203580 further consistently increased myocyte viability to near control levels during treatment with H202 plus hUCBC. See Figure 5. Moreover, inhibition of p38 in intact hearts that are subjected to myocardial ischemia and then reperfusion can reduce myocyte death by as much as 32% by downregulation of BAX protein [66].

In conclusion, the present investigation suggests that hUCBC growth factors and anti-inflammatory cytokines significantly limit the expression of inflammatory cytokines in the myocardium and increase myocyte survival by activating myocyte Akt and downregulating myocyte protein kinases JNK and p38. As a consequence, myocardial infarction size is decreased and significant reductions in ventricular contractility are prevented.

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Conflict of interest

The authors declare no conflict of interest.

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