Original Research

Human cardiac explant-conditioned medium: soluble factors and cardiomyogenic effect on mesenchymal stem cells

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Abstract

The use of conditioned medium (CM) from human cardiac explants (HCEs) as a potential source of paracrine factors for adult stem cell signaling has never been evaluated. We hypothesized that HCEs might provide a source of soluble factors triggering the differentiation of mesenchymal stem cells (MSCs) into cardiomyocyte-like cells. By using two-dimensional electrophoresis (2-DE) gels/mass spectrometry and antibody macroarray assays, we found that HCEs release macromolecules, including cytokines, growth factors and myocardial and metabolism-related proteins into the culture medium. We identified a total of 20 proteins in the HCE-CM. However, as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and 2-DE, these 20 proteins account for only a fraction of the total number of proteins present in the HCE-CM. We also found that CM increased the proliferation of bone marrow-derived-MSCs (BM-MSCs) *in vitro*. Unlike the other effects, this effect was most evident after 48 h of culture. Moreover, we examined the effect of HCE-CM on levels of mRNA and protein for specific cardiac markers. We showed that a surprisingly big fraction of BM-MSCs (3.4–5.0%) treated *in vitro* with HCE-CM became elongated and began to express cardiac markers, consistent with their possible differentiation into cardiomyocyte-like cells. Our *in vitro* model may be useful not only *per se*, but also for studies of the mechanisms of action of soluble factors involved in cell differentiation, paving the way for possible new protein-based treatments in the future.

Keywords: mesenchymal stem cells, conditioned medium, human cardiac explant, cytokines, growth factors

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Introduction

Following myocardial infarction, human cardiomyocytes undergo mitosis to repair the damage. It has also been shown that endogenous or transplanted stem cells contribute to the regeneration of damaged cardiac tissue.[1–4] Nonetheless, major myocardial infarctions lead to replacement of the functional tissue by a fibrotic non-contractile tissue. The proliferative potential of the cardiomyocytes and the number of stem cells capable of differentiating into cardiac myocytes are limited, restricting the potential for complete, functional regeneration after injury. In particular, the regenerative potential is too low to prevent the progression of postinfarction cardiac dysfunctions.[5] Efforts are therefore being made to identify strategies for increasing the myocardial mass after injury.

It has been shown that bone marrow-derived mesenchymal stem cells (BM-MSCs) can differentiate into cardiomyocytes *in vitro* or *in vivo*.[6,7] Several methods have been used to promote the differentiation of stem cells into specific cell types, including cardiomyocyte-like cells. These methods include the co-culture of stem cells with differentiated somatic cells,[8] treatment with cardiac tissue extracts,[9] treatment of MSCs with chemicals such as 5-azacytidine[10] and the treatment of embryonic or adult stem cells with nitric oxide donors.[11,12] Most of these methods are inefficient, resulting in the generation of only a small percentage of differentiated cells after treatment.

In a very different model designed to promote chondrogenic differentiation, it has recently been shown that cartilage explants release soluble signaling factors into the culture medium and that these factors induce the differentiation of adult rat marrow stromal cells into chondrocyte-like cells.[13] The use of conditioned medium (CM) from human cardiac explants (HCEs) as a potential source of

paracrine factors for adult stem cell signaling has never been evaluated. We hypothesized that HCEs might provide a source of soluble factors triggering the differentiation of MSCs into cardiomyocyte-like cells. This system could also be used as a model of cardiac repair *in vitro.*

We identified some of the soluble growth factors and cytokines released by cardiac explants. We also showed that the treatment of BM-MSCs with explants-CM induced the expression of cardiac markers, in terms of both mRNA and protein production, consistent with the putative differentiation of these MSCs into cardiomyocyte-like cells.

Materials and methods

Ethical issues

All human samples used in this work were collected in accordance with guidelines for research involving human subjects and the 1975 Helsinki Declaration. The study was approved by the ethics committee of the Oswaldo Cruz Foundation (approval number 419/07).

Isolation of HCEs and preparation of CM

Cardiac tissue was obtained from the material discarded during valve dissection. Pericardium/epicardium and endocardium were removed and the myocardium (auricle and ventricle) was processed. We cut 600 mg of cardiac tissue into 1 mm^3 pieces, transferred them to a sterile tube and washed them three times with phosphate-buffered saline (PBS) to remove cellular debris. Tissue fragments were dispensed in equal quantities into 75 cm^2 culture flasks (150 mg/flask) containing 20 mL of Iscove's modified Dulbecco's medium (IMDM) without fetal calf serum (FCS) and cultured as explants. Explants were cultured for six days, with complete replacement of the medium every other day. The medium was collected, passed through a filter with 0.70 μm pores (BD Falcon cell strainer) and then through a filter with 0.22 μm pores (thiamine pyrophosphate). The final filtrate was stored at -20°C until use.

Protein sample preparation for two-dimensional electrophoresis

Just before the assay, samples were thawed on ice. Protease inhibitor (1 mmol/L phenylmethylsulfonyl fluoride and 10 μmol/L *N-α-p*-tosyl-L-lysine chloromethyl ketone) was added and total protein was concentrated by precipitation with a 95% saturated solution of $(NH_4)_2SO_4$ and re-suspended in 500 μL of ultra-pure water (18.2 MΩ grade). Excess salts were removed and the buffer was changed by dialyzing the sample for 30 h against focusing buffer (7 mol/L urea, 2 mol/L thiurea, 10 mmol/L Tris-HCl [pH 8] and 10 mmol/L dithiothreitol [DTT]), using a mini-dyalisis kit (GE Healthcare, WI, USA) with a cutoff point of 1 kDa. Before electrophoresis in the first dimension, disulfide bonds were reduced by incubation with 10 mmol/L DTT (45 min at 60°C) and alkylated by incubation with 55 mmol/L iodoacetamide and 10 mmol/L DTT (30 min at room temperature). Protein concentration was determined with the 2D-Quant Kit (GE Healthcare).

Two-dimensional polyacrylamide gel electrophoresis

We solubilized 150 μg of protein in 100 μL of focusing buffer supplemented with 0.5% immobilized pH gradient (IPG) buffer (pH 4–7) (GE Healthcare) and 10% isopropanol. Samples were cup-loaded onto an IPG strip (18 cm, pH 4–7, GE Healthcare) and focused under the following conditions: 50 V–1 h, 100 V–1 h, 300 V–1 h, 500 V–1 h, 1000 V–1 h, 2000 V–1 h, 4000 V–2 h and 6000 V–10 h. For the second dimension, strips were treated for 20 min with equilibration buffer (6 mol/L urea, 30% glycerol, 2% sodium dodecyl sulfate [SDS], 50 mmol/L Tris-HCl [pH 8]) supplemented with 2% DTT and then for 20 min in the same buffer supplemented with 2.5% iodoacetamide. Gels were silver-stained, as described by Blum *et al.*[14]

Matrix-assisted laser desorption/ionization-time of flight/time of flight analysis

Protein spots were manually extracted for mass spectrometry analysis in the 10–70 kDa range. The more intensely stained and well-resolved spots were selected for matrix-assisted laser desorption/ionization-time of flight/time of flight (MALDI-TOF/TOF) analysis. Gel pieces were de-stained, and proteins were reduced (20 mmol/L DTT), alkylated (55 mmol/L iodoacetamide) and digested with 10 ng/μL trypsin (Promega, WI, USA) in 50 mmol/L ammonium bicarbonate. Peptides were extracted (50% acetonitrile, 1% trifluoroacetic acid [TFA]), dried on a Speed Vac and re-suspended in 1 μL of 0.1% TFA for analysis. Peptide sample was mixed 1:1 (v/v) with crystallization solution (5 mg/L 3,5-dimethoxy-4-hydroxycinnamic acid in 50% acetonitrile, 0.1% TFA) and immediately spotted onto a MALDI plate. Mass spectra were obtained by MALDI-TOF/TOF in a mass spectrometer (Autoflex, Bruker Daltonics, Bremen, Germany) operating in positive mode. The Xml extension files containing mass lists were generated with FlexAnalysis software v 2.0 (Bruker Daltonics, MA, USA) and proteins were identified with the Mascot search engine and public databases (NCBI).

Antibody array and analysis

For identification of the growth factors and cytokines released from the HCEs, 1 mL of HCE-CM without FCS (HCE-CM) or non-conditioned IMDM FCS-free (NCM) was assayed with the RayBio™ growth factor and cytokine array (Raybiotech, GA, USA) according to the manufacturer's protocol. Briefly, the membranes were blocked by incubation for 30 min in 5% bovine serum albumin (BSA) in 0.01 mol/L Tris buffer supplemented with 0.15 mol/L NaCl (pH 7.6) and then incubated for two hours in 1 mL of HCE-CM or NCM at room temperature. The membranes were washed and incubated with biotin-coupled anticytokine and antigrowth factor antibodies for two hours, washed again and incubated with horseradish peroxidase-conjugated streptavidin for two hours. We incubated each

membrane with 1 mL of detection reagent (prepared by mixing equal volumes of detection buffers A and B) for 5–10 min and then placed it against X-ray film. Films were developed after various durations of exposure to the membrane (5, 10 and 40 s).

The films were scanned and the data were input into ScanAlyze 2.5 software (www.graphics.stanford.edu/software/scanalyze) for obtaining the raw intensity values of each spot. The normalized intensity of each spot was determined with the Raybio® Analysis Tool. Values were calculated with respect to positive controls and statistical analysis was carried out with GraphPad Prism 5 software. All values are expressed as means ± standard errors of mean and we compared variables between groups by two-way analysis of variance. Values of $P < 0.05$ were considered significant.

Isolation and culture of MSCs

The MSCs used in this study have been isolated from BM and characterized previously by Rebelatto *et al.*[15] Cells were cultured with IMDM (Gibco, NY, USA) supplemented with 15% FCS, 100 U/mL penicillin and 100 μg/mL streptomycin, at 37°C, in a humidified atmosphere containing 5% CO_2. All experiments were performed with MSCs in passage 3.

Culture of MSCs with cardiac explant-CM

Biological triplicates of BM-MSCs were cultured into six-well/plates (8×10^4 cells) and 24-well/plates (4×10^4 cells). The cells were maintained in IMDM supplemented with 15% FCS until they reached 80% confluence. To maintain the same culture conditions for the three biological triplicates of BM-MSCs used in this experiment, the HCE-CM samples were pooled and both media (pooled HCE-CM and NCM) were then supplemented with 5% FCS. After that, BM-MSCs were cultured with a 1 mL pool of either HCE-CM or NCM in six-well plates and a 500 μL pool of HCE-CM or NCM in 24-well plates. The BM-MSCs were cultured for up to three weeks at 37°C, under an atmosphere containing 5% CO_2. The medium was changed every three days. Viable cells were counted, following trypan blue staining, in a Neubauer chamber, for comparison of the numbers of BM-MSCs cultured in HCE-CM and NCM media.

Cell proliferation assay

Cell proliferation assay was performed by bromodeoxyuridine (BrdU) incorporation (100 μmol/L BrdU for 24 h) and labeling according to the manufacturer's recommendations (Invitrogen, NY, USA) with minor modifications. Briefly, the proliferative activity of BM-MSCs was evaluated before and after 48 h of incubation with NCM or HCE-CM. BrdU incorporation was detected using anti-BrdU antibody labeled with Alexa Fluor 488 and analyzed in a FACSCalibur device (BD Bioscience, CA, USA) equipped with CellQuest software. Cell proliferation assays were carried out in duplicate.

Total RNA extraction and reverse transcription-polymerase chain reaction

Total RNA was extracted from cultured MSCs with the RNeasy kit (QIAGEN, TX, USA) according to the manufacturer's instructions. The RNA was treated with DNAse I (QIAGEN) and its concentration was determined with a nanodrop device (ND-100-UNISCIENCE spectrophotometer). We synthesized the first-strand cDNA from 1 μg of total RNA, using 1 μL of 10 mmol/L oligo-dT primer (USB Corporation, OH, USA) and 1 μL of reverse transcriptase (IMRPOM II, Promega) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was carried out with 20 ng of cDNA as the template, 20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 10 pmol of primers, 5 mmol/L of $MgCl_2$, 0.25 mmol/L of dNTPs and 0.6 U *Taq* polymerase (Invitrogen). PCR primer sequences are listed in Table 1. We subjected 20 μL of reverse transcription (RT)-PCR products to electrophoresis in a 2% agarose gel. The bands obtained were visualized by ethidium bromide staining and photographed under ultra-violet transillumination (UV White Darkroom, UVP Biomaging Systems, CA, USA).

Immunofluorescence

Cultured MSCs were washed three times with PBS and fixed by incubation for 5 min in 4% paraformaldehyde. The cells were then permeabilized by incubation for 30 min in 0.5% Triton X-100 in PBS and blocked by incubation for 60 min with 5% PBS-BSA. Blocked cells were

Table 1 Primer sets used for RT-PCR analyses

Gene	Sequence (5′–3′)	Accession number	Amplicon size (bp)	T_M (°C)
GAPDH	F: GGCGATGCTGGCGCTGAGTAC R: TGGTTCACACCCATGACGA	NM 2597	150	55
Cx43	F: CCTTCTTGCTGATCCAGTGGTAC R: ACCAAGGACACCACCAGCAT	NM 65188	154	62
cTnI	F: GGGGGCCCGGGCTAAGGAGTC3 R: AGGGCAGGGGCAGTAGGCAGGAAG	NM 000363.4	183	60
cTnT	F: AGGAGAAGTTCAAGCAGCAGA R: GCGAGCGAGGAGCAGAT	NM 000364.2	155	55
Nkx2.5	F: GGTCTATGAACTGGAGCGGC R: ATAGGCGGGGTAGGCGTTAT	AB021133	322	60
VEGF	F: CTACCTCCACCATGCCAAGTG R: TGCGCTGATAGACATCCATGA	M27281	101	60

RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Cx43, connexin 43; cTnI, cardiac troponin I; cTnT, cardiac troponin T; Nkx2.5, NK2 transcription factor-related, locus 5; VEGF, vascular endothelial growth factor

incubated for one hour at 37 °C with goat anti-human troponin I, goat anti-human troponin T, mouse anti-human α-actinin and mouse anti-human connexin-43 polyclonal antibodies (Santa Cruz Biotechnology, CA, USA) diluted 1:200. The secondary antibodies, Alexa 488-conjugated anti-goat IgG antibody (Invitrogen) diluted 1:400 and Alexa 456-conjugated anti-mouse IgG antibody (Invitrogen), diluted 1:400, were added to the cells, which were then incubated for 30 min. Nuclei were stained by incubation with 4′,6-diamidine-2′-phenylindole (DAPI) for five minutes at room temperature. All the antibodies were diluted in PBS-BSA. Samples were photographed with a Nikon E-600 optical immunofluorescence microscope. For a semi-quantitative analysis of differentiation, the percentages of cells positive for troponins I and T were calculated from 10 random fields (20X), with ImageJ software (http://rsbweb.nih.gov/ij/).

Results

HCE-CM contains cytokines, growth factors, structural and metabolic proteins

Proteins present in the HCE-CM were precipitated and initially analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which showed the sample to be highly complex, containing a wide range of proteins of various sizes, from small polypeptides to high-molecular weight proteins (Figure 1). Despite the thorough washing of the explants before culture, we suspected that some, possibly even most of the proteins seen in the CM, might be the products of explant manipulation and necrosis and/or apoptosis of the pieces of tissue during culture time. A more complete view of the proteome was obtained by subjecting protein samples to two-dimensional gel electrophoresis (2-DE) and analyzing some of the spots obtained by MALDI-TOF/TOF. Gels with narrower pH ranges (4 to 7) gave the best resolution. Most of the significant identifications ($EV < 10^{-3}$) corresponded to structural and metabolism-related proteins (Table 2). The loading of larger amounts of sample would have decreased the resolution, making it harder to identify proteins due to the large number of proteins with molecular weights of up to 70 kDa.

In addition to 2-DE/MALDI-TOF/TOF, we also carried out antibody array analyses to identify the cytokines and growth factors released by the human cardiac tissue. A comparison, by eye, of the HCE-CM and NCM membranes clearly demonstrated that some cytokines and growth factors were present on the HCE-CM membrane, but not on the NCM membrane (Figure 2a). Reproducibility was high for the four samples analyzed. Statistical

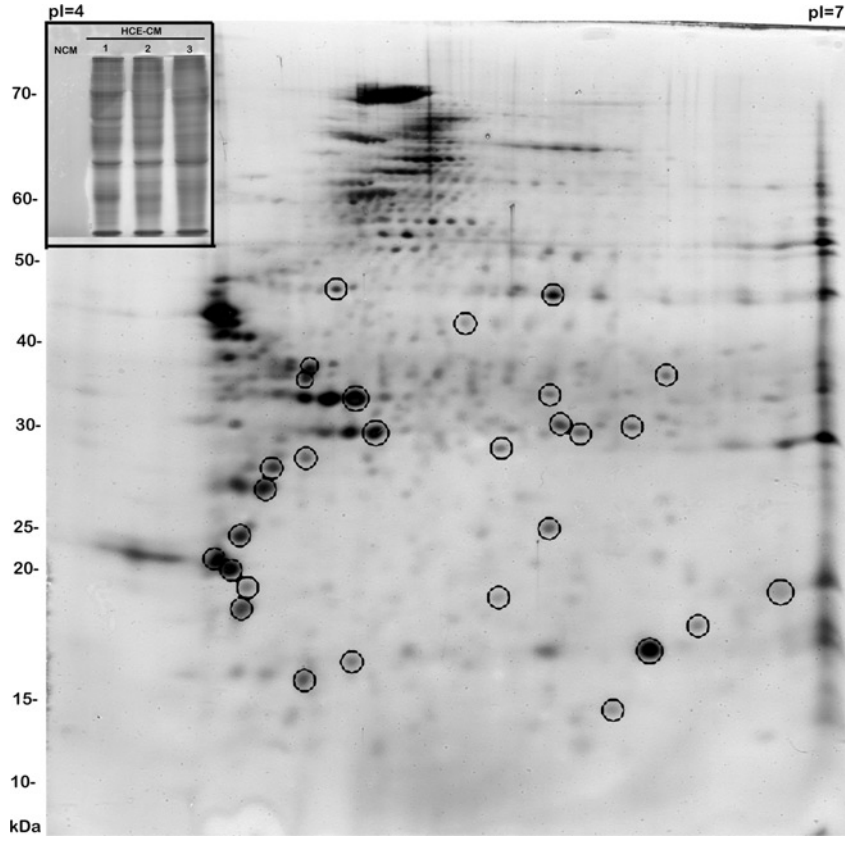

Figure 1 Separation of the proteins present in HCE-CM by SDS-PAGE and 2-DE. The proteins present in HCE-CM were precipitated and analyzed by SDS-PAGE (15% polyacrylamide gel). Numbers 1, 2 and 3 correspond to the biological replicates (inserted top left panel). The proteins in the HCE-CM were also analyzed by 2-DE. Assays were performed in two different pI ranges (3–10 and 4–7). A representative gel is shown for the pI 4–7 range. Indicated spots (circled spots) were excised and subjected to in-gel digestion with trypsin. The resulting peptides were identified by MALDI-TOF/TOF (see Table 2). NCM, non-conditioned medium; HCE-CM, human cardiac explant-conditioned medium; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; 2-DE, two-dimensional electrophoresis

Table 2 Proteins identified in the CM by 2-DE

Protein	EV	Gene ID
TPI1	5.60E − 13	7167
ACTN3	3.50E − 08	89
MDH1	8.00E − 05	4190
DCI	3.80E − 04	1632
MyBPC	4.20E − 04	4607
ACTA2	5.50E − 05	59
SOD2, mitochondrial	5.70E − 04	6648
ACTC1	1.00E − 03	70
MYL4	4.00E − 03	4635
MYLK	5.90E − 03	4638

CM, conditioned medium; 2-DE, two-dimensional electrophoresis; EV, expected value; TPI1, triose phosphate isomerase 1; ACTN3, actinin, alpha 3; MDH1, malate dehydrogenase 1; DCI, dodecenoyl-coenzyme A delta isomerase; MyBPC, cardiac myosin binding protein C; ACTA2, actin, alpha 2, smooth muscle; SOD2, superoxide dismutase 2; ACTC1 actin, alpha, cardiac muscle 1; MYL4, myosin, light chain 4; MYLK myosin light chain kinase

analysis showed that four of the 23 detectable cytokines on the antibody arrays were present in significant amounts in the CM ($P \leq 0.05$). These cytokines were as follows: growth-regulated oncogene (GRO), interleukin-6 (IL-6), IL-8 and monocyte chemoattractant protein-1 (MCP-1) (Figure 2b). Six of the 41 growth factors detectable on the antibody arrays were present in significantly larger amounts ($P \leq 0.01$) in the HCE-CM. These growth factors were: basic-fibroblast growth factor (bFGF), epidermal growth factor (EGF), hepatocyte growth factor (HGF), insulin-like growth factor binding protein-1 (IGFBP-1), IGFBP-2 and transforming growth factor-β (TGF-β) (Figure 2c). The fluorescence intensity of the spots on the HCE-CM membranes ranged from approximately 1.5 to approximately 12 times the background signal (NCM). For example, IL-6 signal intensity on HCE-CM membranes was almost 10 times that on NCM membranes, whereas IL-8 signal intensity on HCE-CM membranes was only 1.5 times higher than the background signal, although this difference was nonetheless significant ($P < 0.05$).

These results suggest that HCE-CM is a source of paracrine factors, such as cytokines, growth factors and other proteins and unidentified molecules.

HCE-CM increases the proliferation of BM-MSCs in culture and induces the differentiation of these cells into cardiomyocyte-like cells *in vitro*

The total number of viable BM-MSCs was higher in the HCE-CM-treated cultures than in the NCM-treated cultures as established by trypan blue staining and counting in Neubauer chamber. To determine if this increase in cell number was caused, at least in part, by an increase in cell proliferation, BrdU incorporation experiments were carried out. Following the treatment of BM-MSCs with HCE-CM for 48 h, the percentage of proliferating BM-MSCs was 1.5- to 1.8-fold higher than in the NCM-treated cells (Figure 3). The result from two BM-MSCs samples were plotted individually due to the different cell cycle kinetics seen between patients (Figure 3a). Proliferation increase in HCE-CM-treated cells is quite evident after 48 h in the representative FACS histograms (Figure 3b) and also in microscopy images of the cultures (Figure 3c).

We also examined the effect of HCE-CM, during the first and third weeks of treatment, on levels of mRNA and protein for specific cardiac markers (troponins I and T, α-actinin, Nkx 2.5, connexin-43) and an angiogenic factor (vascular endothelial growth factor [VEGF]) in BM-MSCs. Most of the effects of CM on BM-MSCs were evident after one week of culture, but were much more marked at the third week, for the three BM-MSCs replicates analyzed. RT-PCR was used to determine mRNA levels. Troponin T, troponin I, NKx 2.5, connexin-43 and VEGF transcript levels were higher in all HCE-CM-treated samples than in NCM-treated cells. However, BM-MSCs spontaneously expressed some of the genes analyzed, to some extent (Figure 4).

Indirect immunofluorescence analyses of protein distribution in BM-MSC cultures demonstrated clear differences between treated and untreated samples. After one week of culture, a subpopulation of cells positive for troponin I (Figure 5a and e), troponin T (Figure 5c and g) and α-actinin (Figure 5d and h) was detected in all the HCE-CM-treated samples but not in the NCM-treated samples. Most of the cells positive for these cardiac markers were spindle shaped, with elongated nuclei, as shown by DAPI staining (Figure 5). As stated above, cardiac markers were more strongly expressed after three than after one week, whether we considered the absolute numbers of cells expressing these markers or the fluorescence intensity of the elongated cells (Figure 5i, k, l, m, o and p). Again, the control cultures (treated with NCM) also included cells positive for these markers, but in much smaller numbers than for the HCE-CM-treated cultures. Differently to these markers, connexin-43 was present in most of the NCM- and HCE-CM-treated cells. Nevertheless, connexin-43 staining was seen in the boundaries between adjacent elongated cells in the cultures treated with HCE-CM for three weeks.

A semi-quantitative analysis of cells positive for troponins T and I showed that HCE-CM-treated BM-MSC cultures contained four times as many positive, spindle-shaped cells with elongated nuclei than NCM-treated cells. The percentage of cells reacting with antibodies against troponins I and T was determined by analyzing 10 random fields from each sample and for each marker. After one week, the mean percentages of cells positive for troponins I and T were 3.4 ($\pm$1.3) and 3.9 ($\pm$1.7), respectively. After three weeks of treatment with HCE-CM, the mean percentages of cells immunopositive for troponins I and T were 4.7 ($\pm$1.7) and 5.0 ($\pm$2.2), respectively. The ratio of the proportion of positive HCE-CM- to NCM-treated cells was about 4.0 in all cases, with no significant differences between markers or weeks of treatment. These results suggest that the stronger effects of HCE-CM after three weeks than after one week result from a larger absolute number of positive cells rather than an increase in the percentage of immunopositive cells.

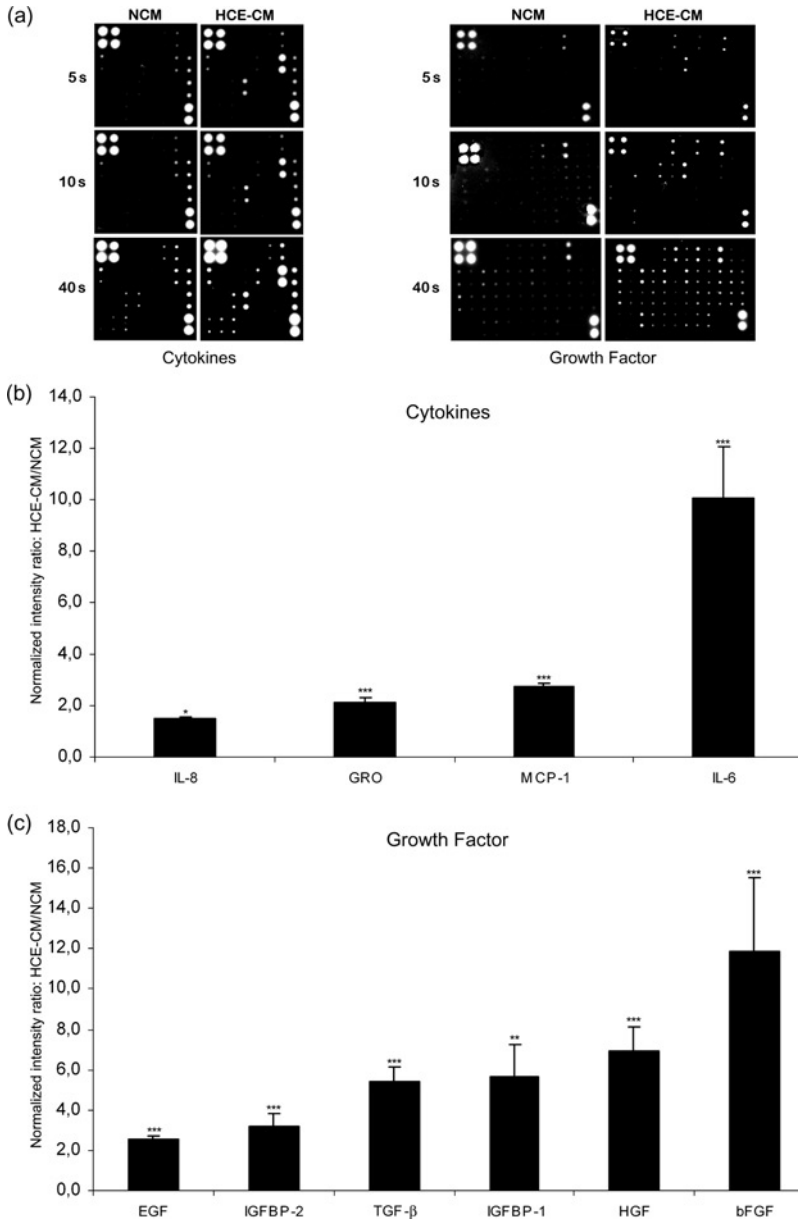

Figure 2 Growth factors and cytokines present in HCE-CM. (a) Antibody array analysis. Membranes were placed against X-ray film for various durations (5, 10 and 40 s). The film was then developed and the image was scanned and input into Scanalyze software for analysis. Values for each spot were calculated and normalized by subtracting the background signal from blank spots and dividing by the mean value for positive controls. Eight values were generated for each factor, from four biological replicates and four technical duplicates. Error bars indicate the standard error for the four biological replicates. (b) Graphical representation of cytokines and (c) growth factors present in significant amounts ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$) in HCE-CM, as determined by comparison with the background signal generated for NCM membranes (*y*-axis – normalized intensity ratio: HCE-CM/NCM). Error bars represent the standard error for the four biological replicates. HCE-CM, human cardiac explant-conditioned medium; NCM, non-conditioned medium; TGF-β, transforming growth factor-β; IL, interleukin; GRO, growth-regulated oncogene; MCP, monocyte chemoattractant protein; EGF, epidermal growth factor; IGFBP, insulin-like growth factor binding protein; HGF, hepatocyte growth factor; FGF, fibroblast growth factor

Discussion

We found that HCE release macromolecules, including cytokines, growth factors and myocardial and metabolism-related proteins into the culture medium. We identified a total of 20 proteins in the cardiac explant-CM. However, as shown by SDS-PAGE and 2-DE, these 20 proteins account for only a fraction of the total number of proteins present in the HCE-CM. A high-throughput approach is required for full characterization of the CM. We are currently working on a proteomics project aiming to generate an exhaustive list of the proteins present. We also found that CM increased the number of treated BM-MSCs *in vitro* at least partially due to induced proliferation. It remains unclear as to whether HCE-CM also has a cytoprotective/antiapoptotic effect. Furthermore, we showed that a fraction of BM-MSCs treated *in vitro* with HCE-CM became elongated and began to express cardiac markers, consistent with their possible differentiation into cardiomyocyte-like cells.

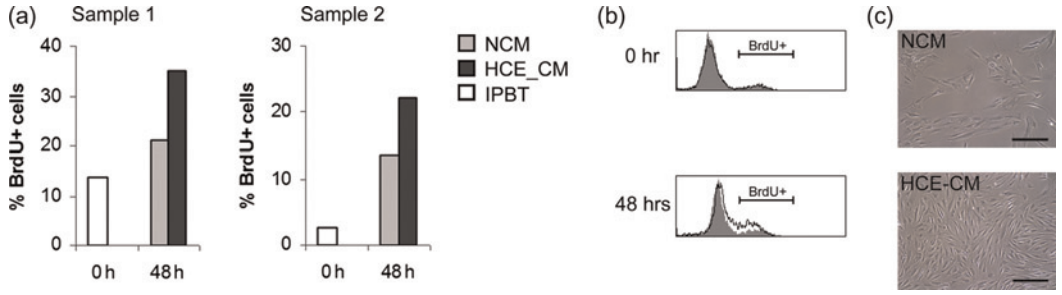

Figure 3 Effect of conditioned medium on MSC proliferation. (a) Graphs showing the percentage of BrdU-positive proliferating cells on two different samples of BM-MSC cultured in HCE-CM or NCM for 48 h. The open bar represents the proliferation rate of the initial cell population (0 h) of both BM-MSCs samples before NCM or HCE-CM treatment (IPBT). (b) Representative histograms of BrdU-positive proliferating cells cultured in HCE-CM (open histogram) or NCM (gray area) at 0 and 48 h. (c) Representative images (differential interference contrast (DIC) microscopy) of BM-MSCs cultured with NCM and HCE-CM for 48 h. Scale bar: 30 μm. MSC, mesenchymal stem cells; BrdU, bromodeoxyuridine; BM, bone marrow; HCE-CM, human cardiac explant-conditioned medium; NCM, non-conditioned medium; IPBT, initial population before treatment

The heart is a complex organ consisting of several different cell types and tissues. Cardiomyocytes are the main cellular component of the heart, but non-muscle cells such as endothelial cells, mesenchymal cells, fibroblasts, smooth muscle cells and leukocytes are also present. All these cells are potential sources of paracrine factors that might help to repair cardiac tissue after injury (cell growth and differentiation). Adult, resident stem cells have also been found in the heart. These undifferentiated cells reside, in extremely small numbers, among the differentiated cells in mature organs or tissues.[16] They have been shown to produce and secrete a wide range of cytokines, chemokines and growth factors potentially involved in cardiac repair.[17] In this context, we hypothesized that cardiac explants might release a set of factors participating in cardiac repair.

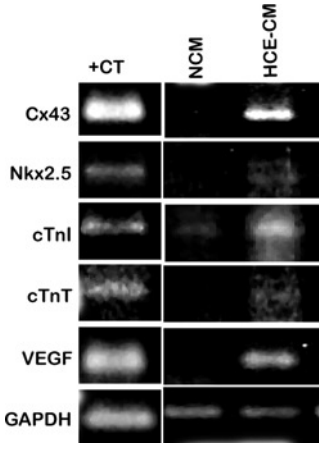

Figure 4 Gene expression profiling by RT-PCR. RT-PCR analysis of mRNA levels for cardiac markers. Representative results of three independent experiments, after 21 d of culture, are shown. We used mRNA isolated from NCM-treated cells as a negative control and mRNA isolated from cardiac tissue as positive control (+CT). GAPDH was used as an internal control. Cx43, connexin 43; Nkx2.5, NK2 transcription factor-related, locus 5; cTnI, cardiac troponin I; cTnT, cardiac troponin T; VEGF, vascular endothelial growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription-polymerase chain reaction; NCM, non-conditioned medium; HCE-CM, human cardiac explant-conditioned medium

Using explant culture, a technique for the isolation of cells from fragments of body tissue, Barile *et al.*[18] showed that left atrial or ventricular biopsy specimens could generate a layer of migratory cells. In this study, we used tissue fragments exclusively as a source of paracrine factors. We cultured the explants for six days to condition the medium, and we observed no cell migration from them. Cell migration from cardiac explants takes at least two weeks of culture.[4] However, we nonetheless continued to culture the explants for four weeks, to check that they were viable. In fact, all the explants used to condition the medium in this study were viable and different cells had migrated from them after two weeks of culture (data not shown).

Structural (cMyBP-C and α-actin) and metabolic (triose phosphate isomerase and malate dehydrogenase) proteins were detected by 2-DE/MALDI-TOF/TOF. Despite thorough washing of the explants before culture, these proteins were probably present as a result of the mechanical dissociation required to obtain cardiac explants or due to the normal necrotic/apoptotic process occurring during culture. Several studies have identified candidate biomarkers for the analysis of proteins released into the blood after myocardial injury. Structural myocardial proteins, such as the cardiac troponins, have been detected in the bloodstream and are currently used as serum markers for myocardial cell necrosis and/or myocardial ischemia. Moreover, the concentrations of these proteins in the serum are associated with the severity of the injury.[19–21] MyBP-C has been detected in mouse hearts subjected to ischemia reperfusion,[22] in dog hearts subject to low-flow rate ischemia[23] and in the plasma of mice with acute myocardial infarction.[24] The study on mice with myocardial infarction anticipated cMyBP-C as an excellent new candidate biomarker of infarction, due to the high levels of this protein found in the bloodstream after injury and the rapidity of the response. The presence of cMyBP-C and other non-secreted proteins in HCE-CM suggests that our experimental model may be of potential value for use in studies of cardiac injury and repair mechanisms.

Antibody array analysis demonstrated the presence of many secreted cytokines and growth factors in HCE-CM.

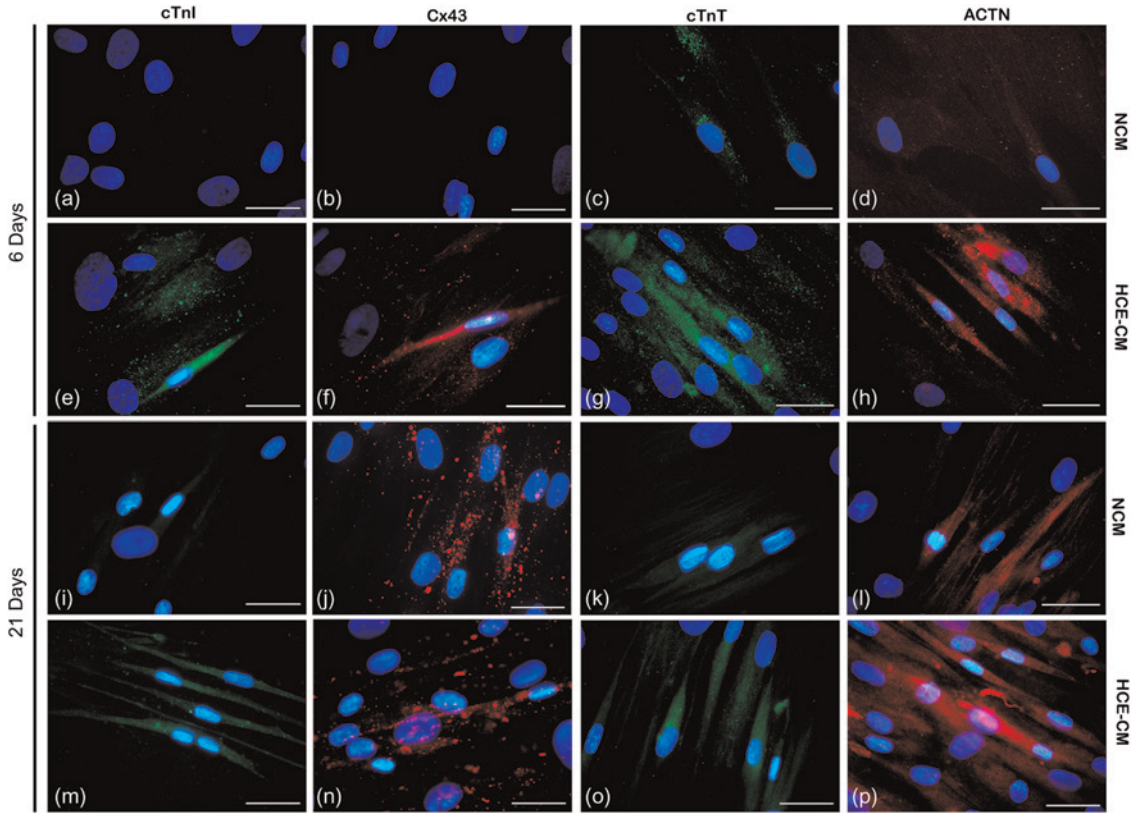

Figure 5 Indirect immunofluorescence analysis. Indirect immunofluorescence analysis of the expression of cardiac markers. BM-MSCs were treated with NCM (a–d; i–l) and HCE-CM (e–h; m–p) for six days (a–h) and 21 d (i–p). Immunofluorescence analyses were performed for the markers indicated at the top. Days of treatment are shown on the left and the medium used for MSC culture is shown on the right (HCE-CM or NCM). Cell nuclei are stained with DAPI (blue). Representative results from three independent experiments are shown. Scale bar = 30 μm. Cx43, connexin 43; cTnI, cardiac troponin I; cTnT, cardiac troponin T; ACTN, α-actinin; BM-MSC, bone marrow-derived mesenchymal stem cells; NCM, non-conditioned medium; HCE-CM, human cardiac explant-conditioned medium; DAPI, 4′,6-diamidine-2′-phenylindole (A color version of this figure is available in the online journal)

One of these molecules, IL-6, has been shown to be released in response to the stress caused by myocardial injury.[25] In the acute phase of myocardial infarction, inflammatory activation is enhanced, with a predominantly proinflammatory response and high serum concentrations of IL-6.[26] IL-6 is a pleiotropic cytokine responsible for many different processes, including the regulation of cell growth, apoptosis, survival and differentiation in various cell types and organs, including the heart.[27] The local or systemic production of inflammatory mediators may influence not only MSC migration to injured tissues,[28–30] but also MSC proliferation, differentiation[31] and engraftment.[32]

Moreover, cytokines, such as IL-6, can induce the secretion of important chemokines involved in the regulation of angiogenesis.[33] The IL-8, GRO and MCP-1 chemokines are also detected in the HCE-CM. The healing of the myocardium after infarction involves changes in the adhesive interactions between endothelial cells in the preexisting vasculature and extracellular matrix, under the control of locally produced factors (cytokines, chemokines). These changes lead to endothelial cell migration, proliferation, re-organization and microvessel formation.[34] Indeed, the IL-6 released into the HCE-CM may induce the secretion of MCP-1, IL-8 and GRO into the medium. Kocher *et al.*[34] found that the production of IL-8 and GRO by endothelial cells regulated the ability of chemokines to induce myocardial neovascularization, and mediated protection against cardiomyocyte apoptosis and the recovery of cardiac function. The cytokines present in HCE-CM may therefore work together to repair the myocardial injury caused by the cutting of cardiac tissue, by increasing the number of cells or inducing cardiomyocyte differentiation.

Growth factors, such as bFGF, EGF, HGF, IGFBP-1 and -2 and TGF-β, were also present in HCE-CM. Some of these factors play a role in tissue repair and, as reported previously, are also crucial for cardiomyocyte specification during development. It has also been shown that FGF, EGF, TGF and IGFBP play a role not only in embryo development, but also in the cardiogenic potential of embryonal carcinoma cells in culture.[35,36] TGF-β is a multipotent factor associated with neoangiogenesis, decreasing inflammatory signals and inducing MSC differentiation. Li *et al.*[37] reported the induction by TGF-β of the differentiation of BM-MSC into immature cardiomyocytes, and Abarbanell *et al.*[38] reported a correlation between prolonged exposure to TGF-β and HGF *in vitro* with cardiac surface marker expression on BM-MSCs. Similarly, we showed that HCE-CM containing TGF-β, HGF, bFGF and IGFBP-1 and -2 induced an increase in cardiac marker expression, as indicated by RT-PCR and immunofluorescence assays. Zhu *et al.*[36] have shown that IGFBP-4 induces cardiomyocyte differentiation by antagonizing Wnt/β-catenin and,

other members of the IGFBP family, such as IGFBP-1 and -2, are genetically redundant with IGFBP-4. Accordingly, we are currently studying how IGFBP-1 and -2 might affect proliferation and cardiomyocyte differentiation of BM-MSCs in culture.

NCM-treated BM-MSCs also produced mRNA and protein for some cardiac markers. The spontaneous expression of muscle markers by MSCs has been reported before,[10,39–41] and Verfaille's group described MSCs as 'pluridifferentiated' cells.[42] Alternatively, as it was observed in this work, it might be due to the spontaneous differentiation of a small fraction of cells.

In conclusion, this study demonstrates that HCEs release cytokines and growth factors into the medium and that these molecules induce the proliferation and differentiation of human BM-MSCs into cardiomyocyte-like cells. Moreover, our *in vitro* model may be useful for studies of the mechanisms of action of soluble factors involved in cell differentiation, paving the way for possible new protein-based treatments in the future.

Author contributions: AVS and AC conceived and designed the experiments and wrote the paper; AVS performed the experiments; AVS, PFC and MK performed the 2-DE experiments; AVS, MAS and AC interpreted the studies and analyzed the data; FDAC and PH supplied biological samples and assisted with BrdU experiments; and SG and BD analyzed the data and reviewed the manuscript.

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