Mechanisms of paracrine cardioprotection by cord blood mesenchymal stromal cells

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BASIC SCIENCE

Abstract

OBJECTIVES: Among the mechanisms by which somatic stem cells may improve left ventricular function in ischaemic heart disease are pro-survival stimuli mediated by secreted factors. This phenomenon is frequently referred to, but remains poorly understood. We therefore investigated the non-regenerative cardioprotective effects of cord blood mesenchymal stromal cells (CBMSCs) in vitro and sought to identify relevant intracellular signalling pathways.

METHODS: Conditioned medium from CBMSCs and fibroblasts was prepared, and secreted factors were analysed by Luminex® immuno-bead assay. Murine cardiomyocyte-derived HL-1 cells were subjected to simulated ischaemia by glucose and serum deprivation and hypoxia in CBMSC-conditioned or cell-free control medium or in medium conditioned by foreskin fibroblasts. The proportions of vital, apoptotic and necrotic cells (poly-caspase activity, annexin V and ethidium homodimer-III staining) were quantified using a high-content imaging system. Metabolic activity and proliferation rate were determined via 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium and 5-bromo-2-deoxyuridine assays. Phosphorylation of Akt, extracellular-signal-regulated kinase (ERK) 1/2, signal transducer and activator of transcription 3 (STAT3) and glycogen synthase kinase 3β was determined by western blot, and experiments were repeated in the presence of specific small-molecule inhibitors ( Wortmannin, UO126 and Stattic).

RESULTS: CBMSC medium reduced the proportion of dead HL-1 cardiomyocytes from 39 ± 3 to 28 ± 1% (P < 0.05) and the rate of late apoptotic cells to 68 ± 2% of that in control medium (P < 0.001). Metabolic activity was increased by 12 ± 1% compared with control (P < 0.05), while in fibroblast medium it was not (5 ± 2%, P = 1). This was associated with increased phosphorylation of Akt (2-fold, P < 0.05), ERK1/2 (3-fold, P < 0.01) and STAT3 (12-fold, P < 0.001). Combined blocking of the phosphatidylinositol-4,5-bisphosphate 3-kinase/Akt and mitogen-activated protein kinase/ERK signalling abolished the protective CBMSC effect, while blocking the pathways individually had no effect. Inhibition of STAT3 phosphorylation drastically lowered HL-1 cell viability in control medium, but not in medium conditioned by CBMSCs.

CONCLUSIONS: The factors released by CBMSCs protect cardiomyocyte-like HL-1 cells from simulated ischaemia more than those released from fibroblasts. While CBMSC-triggered Akt and ERK1/2 activation provides protection in a compensatory manner, STAT3 is crucial for cardiomyocyte survival in ischaemia, but is not a key mediator of cytoprotective stem cell actions.

Keywords: Ischaemia • Cell therapy • Cardiomyocyte • Stem cell • Cord blood

INTRODUCTION

Numerous pre-clinical studies have demonstrated beneficial stem cell actions on injured myocardium and examined diverse concepts of repair and regeneration. Since improved heart function is seen without trans-differentiation of implanted cells, it has been proposed that paracrine effects mediated by secreted factors favourably influence myocardial perfusion, remodelling and cell survival [1]. While the induction of angiogenesis by soluble factors has been extensively studied, the cytoprotective effects on ischaemic cardiomyocytes are still poorly understood. Some information is available from studies using unfractionated human bone marrow cell products which contain numerous poorly defined subpopulations of cells [2]. For instance, Yasin [3] demonstrated that both rat bone marrow mononuclear cells and their supernatant attenuate in vivo ischaemia/reperfusion injury. Lately, there has been a focus on mesenchymal stromal/stem cells (MSCs), a more uniform cell population with low immunogenicity and robust behaviour in culture. Clinical trials showed functional benefits of MSCs after acute myocardial infarction [4], and their capability to reduce apoptosis of hypoxic/ischaemic cardiomyocytes has been demonstrated in vitro, ex vivo and in vivo [5–7]. To better
understand this phenomenon, we now studied the role of typical cardioprotective signalling cascades [phosphatidylinositol-4,5-bisphosphate 3-kinase/Akt (PI3K/Akt), mitogen-activated protein kinase/extracellular-signal-regulated kinase (MEK/ERK) and signal transducer and activator of transcription 3 (STAT3) pathways]. For this purpose, we established an in vitro model of simulated ischaemia using the murine cardiomyocyte-derived cell line HL-1 and studied the cytoprotective effect of CBMSC- or fibroblast-conditioned medium.

MATERIALS AND METHODS

Cells and cell culture

Murine HL-1 cardiomyocytes, generously provided by Dr William C. Claycomb, New Orleans, USA, were maintained in Claycomb medium with 10% foetal bovine serum (FBS), 2 mM L-glutamine, 100 μM norepinephrine, 100 U/ml penicillin and 100 μg/ml streptomycin, and cultured on gelatine (0.02%) and fibronectin (5 μg/ml)-coated flasks at 37°C and 5% CO₂ in a humid atmosphere (all Sigma-Aldrich, Taufkirchen, Germany). Twenty-four hours prior to the experiments, cells were plated in culture plates (density 1.3 × 10⁵/cm²). Cryopreserved human umbilical cord blood mesenchymal stromal cells (CBMSCs) were provided by Karen Bieback, who isolated and expanded them according to a previously published protocol [8]. Cord blood was obtained with informed consent of the mother, according to the principles of the Declaration of Helsinki and approved by the local ethics committees (Ref. 48/05 reconfirmed in 2009). With a seeding density of 700–1000 cells/cm² and harvesting at subconfluency, MSCs were expanded to the fourth passage in Dulbecco’s Modified Eagle Medium (DMEM) 21885 (low glucose), supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C and 5% CO₂ in a humid atmosphere (all Life Technologies, Darmstadt, Germany). The ability of CBMSCs to differentiate into non-haematopoietic cell types was ascertained (see Supplementary Material), and we previously confirmed their immunophenotype [9]. CRL-2429™ fibroblasts from human foreskin were purchased from LGC Standards, Wesel, Germany, and cultured according to American Type Culture Collection (ATCC®) protocols.

Simulated ischaemia model

To simulate ischaemic conditions in vitro, HL-1 cardiomyocytes were subjected to combined oxygen, glucose and serum deprivation. Unless otherwise stated, cells were placed for 5 h in DMEM 11966 (no glucose) without FBS in a hypoxic atmosphere (1% O₂/5% CO₂), achieved by replacing oxygen with nitrogen in an O₂- and CO₂-controlled incubator. When varying combinations of oxygen, glucose and serum deprivation were tested, standard culture medium was replaced by fresh DMEM 31966 (no glucose) with or without 10% FBS (all Life Technologies) in hypoxia or normoxia. Throughout this paper, the combined oxygen, glucose and serum deprivation is referred to as ‘simulated ischaemia’. In the validation experiments, ‘hypoxia’ refers to 1% O₂ alone, independent of the presence of glucose and serum.

Preparation of conditioned medium

Conditioned medium was prepared using CBMSCs in passage 4 and fibroblasts between passages 10 and 12. Cells were plated at 10 000 cells/cm². After 6 ± 1 (CBMSC) and 3 ± 1 (fibroblasts) days, at 80–90% confluence, cells were washed twice with Dulbecco’s phosphate buffered saline (DPBS) and covered with DMEM 11966 (no glucose), supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (all Life Technologies). After 6 days’ incubation in a 1% O₂/5% CO₂ atmosphere, viability by trypan blue exclusion was 68 ± 4% for CBMSCs and 62 ± 3% for fibroblasts (P = 0.3) (Supplementary Fig. 2). Conditioned medium was collected and centrifuged for 10 min at 300 × g to remove detached cells. To account for possible cardioprotective effects of the medium alone, equivalent medium kept under the same conditions in flasks without cells was prepared as control. Concentration of medium by volume reduction was not performed. In preliminary experiments, medium conditioned for shorter periods of time had less cardioprotective activity, while additional filtration of the medium had no impact on the results (data not shown).

Analysis of conditioned medium

Total protein content in conditioned media was measured with the Roti®-Nanoquant assay (Karl Roth, Karlsruhe, Germany). For quantification of growth factors and cytokines, a Luminex® Screening Assay (R&D Systems, Wiesbaden, Germany) for epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), interleukin (IL)-6, IL-10, tumour necrosis factor (TNF)-α and Angiopoetin-2 was performed using the Bio-Plex® 200 System (Bio-Rad, München, Germany).

Evaluation of cell damage

HL-1 cells were incubated with poly-caspase substrate sulphorhodamine-Val-Ala-Asp-fluoromethylketone (SR-VADEMK) (Biomol, Hamburg, Germany) after simulated ischaemia in 96-well imaging plates (PerkinElmer, Rodgau-Jügesheim, Germany). After fixation with 4% paraformaldehyde (PFA), nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Total remaining cell number, caspase-active cells and nuclear shrinking and fragmentation were quantified using the high-content imaging system Operetta™ and Harmony™ software (PerkinElmer, Waltham, MA, USA). The coefficient of variation of nuclear stain fluorescence intensity was the nuclear fragmentation index (NFI). For calculation of proportions of detached dead cells and adherent caspase-active and non-active cells, data were normalized to the mean cell number in plates not subjected to simulated ischaemia. Detached cells were subjected to trypan blue exclusion and proved to be 100% non-viable. In separate experiments, the Apoptotic/Necrotic Cells Detection Kit (PromoKine, Heidelberg, Germany) was used to further characterize the remaining adherent cells by staining with annexin V (AnnV)-fluorescein isothiocyanate (FITC) and ethidium homodimer (EthD)-III. Then, cells were fixed with 4% PFA, nuclei were counterstained with DAPI and analysed with the Operetta™ system. For the determination of viability/metabolic activity after simulated ischaemia, cells were incubated for 4 h at normoxia in medium...
containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Promega, Mannheim, Germany) and phenazine methosulphate (PMS) (Sigma-Aldrich). Absorbance at 490 nm was measured using a standard microplate reader. Proliferation was determined by 5-bromo-2-deoxyuridine (BrdU) uptake using Cell Proliferation ELISA (Roche Diagnostics, Mannheim, Germany). After simulated ischaemia, cells were incubated for 2 h under normoxic conditions in medium containing BrdU. Then, cells were processed according to the manufacturer’s instructions. Absorbance at 370 and 492 nm (reference) was measured using a standard microplate reader.

Western blotting

Cells were washed with DPBS and lysed in sodium dodecyl sulphate (SDS) buffer with Complete proteinase inhibitor cocktail and PhosSTOP phosphatase inhibitor cocktail tablets (Roche Diagnostics). Protein concentration was determined by bicinchoninic acid (BCA) protein assay (Thermo Scientific, Bonn, Germany), and denatured protein (35 μg) was resolved in a 12% polyacrylamide SDS gel and transferred onto a nitrocellulose membrane (Karl Roth). Membranes were blocked and incubated with monoclonal mouse anti-total protein and rabbit anti-phospho-protein primary antibodies overnight at 4°C: Akt, phospho-Akt (Ser473), ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), STAT3, phospho-STAT3 (Tyr705), glycogen synthase kinase 3β (GSK3β), phospho-GSK3β (Ser9), phospho-STAT1 (Tyr701), phospho-STAT5 (Tyr694) (all New England Biolabs, Frankfurt, Germany), and denatured protein (35 μg) was resolved in a 12% polyacrylamide SDS gel and transferred onto a nitrocellulose membrane (Karl Roth). Membranes were blocked and incubated with monoclonal mouse anti-total protein and rabbit anti-phospho-protein primary antibodies overnight at 4°C: Akt, phospho-Akt (Ser473), ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), STAT3, phospho-STAT3 (Tyr705), glycogen synthase kinase 3β (GSK3β), phospho-GSK3β (Ser9), phospho-STAT1 (Tyr701), phospho-STAT5 (Tyr694) (all New England Biolabs, Frankfurt, Germany). After 1 h incubation with IRDye® conjugated secondary antibodies (Li-Cor Bioscience, Bad Homburg, Germany), blots were analysed using the infra-red imaging system and software Odyssey® (Li-Cor Bioscience).

Statistical analysis

Results are expressed as mean ± SEM. The significance of intergroup differences was determined by one-way analysis of variance (ANOVA) with Bonferroni correction for multiple comparisons. Time-related changes were analysed by constructing a general linear model with univariate ANOVA with Bonferroni correction. When only two groups were compared, a two-tailed t-test was performed. PASW statistics 18, IBM SPSS, was used for data analysis. A P-value of <0.05 was considered significant. A detailed list of the number of experiments per assay is given in the Supplementary Material.

RESULTS

Validation of the simulated ischaemia model in HL-1 cells

HL-1 cardiomyocytes were subjected to combined oxygen, glucose and serum deprivation (simulated ischaemia) for 1–5 h. Within this timeframe, the proportions of poly-caspase-active and detached dead cells increased progressively (Fig. 1A), in parallel to nuclear shrinking and fragmentation in attached cells (Fig. 1B and C). The cell damage was also reflected by decreasing MTS conversion rate. After 5 h, 23 ± 4% of the cells were poly-caspase-active, 30 ± 3% were detached and dead (P < 0.001) (Fig. 1A) and the MTS conversion rate was 66 ± 3% of that of cells not subjected to simulated ischaemia (P < 0.01) (Fig. 1D). To further standardize the simulated ischaemia model, we assessed the impact of its individual factors by subjecting HL-1 cardiomyocytes in parallel experiments to varying combinations of oxygen, glucose and serum deprivation. In normoxia, after 5 h single or combined withdrawal of glucose and FBS, no increase in poly-caspase-active cells was detected. Similarly, after 5 h hypoxia, but in the presence of glucose and FBS (full medium), the percentage of poly-caspase-active cells was not different from that in normoxia (0.5 ± 0.1 vs 0.6 ± 0.1%, P = 1). Sole withdrawal of FBS in hypoxia did not affect the percentage of apoptotic cells, either, while sole glucose withdrawal led to an increase of poly-caspase-active cells by 10 ± 0.6% (P < 0.01 vs full medium in hypoxia). Finally, when subjected to combined oxygen–glucose–serum deprivation, 21 ± 3% of the remaining attached cells became positive for poly-caspase activity (P < 0.001 vs full medium in hypoxia) (Fig. 1E). We also quantified the number of remaining attached cells, which were either viable or undergoing apoptosis, because dead HL-1 cells rapidly detached and floated in the supernatant. In the presence of glucose and FBS, the total cell number was similar after 5 h hypoxia or normoxia (5 ± 0.3 × 10^4 vs 4.5 ± 0.2 × 10^4, P = 1), and this was also the case when only FBS or glucose was withdrawn. However, combined glucose–serum deprivation in hypoxia reduced the number of attached cells to 3.6 ± 0.3 × 10^4 (P < 0.01 vs full medium in hypoxia) (Fig. 1F). Analysis of nuclear morphology also revealed that nuclear shrinking and fragmentation were most increased under combined oxygen–glucose–serum deprivation (Fig. 1G and H). In summary, combined withdrawal of oxygen, glucose and serum for up to 5 h resulted in a magnitude of apoptosis and cell death that proved to be suitable for our experiments, because there was sufficient room for both improvement and further deterioration after treatment with CBMSC-conditioned medium.

Growth factors and cytokines in conditioned medium

In fibroblast-conditioned medium, the total protein content was 26 ± 10% lower than in CBMSC medium (CBMSC 27 ± 2 vs fibroblasts 20 ± 2 μg/ml, P < 0.05). The concentrations of selected growth factors and cytokines are shown in Fig. 2. The concentrations of VEGF (CBMSC 6.0 ± 0.9; fibroblasts 4.9 ± 0.7 pg/ml), EGF (CBMSC 0.83 ± 0.09; fibroblasts 0.65 ± 0.06 pg/ml) and Angiopoietin-2 (CBMSC 0.44 ± 0.04; fibroblasts 0.49 ± 0.06 pg/ml) were similar, CBMSC medium contained more HGF (CBMSC 0.36 ± 0.05 vs
Figure 1: Apoptosis and cell loss of HL-1 cardiomyocytes during 5 h simulated ischaemia. (A) Proportions of detached dead cells and attached poly-caspase-active and non-active cells expressed as the percentage of the non-ischaemic control (*P < 0.001 vs non-ischaemic control; n = 8 triplicates). (B) Mean nuclear area and (C) NFI. (D) Cell viability determined by MTS conversion, expressed as the percentage of the non-ischaemic control (*P < 0.05 and **P < 0.01 vs non-ischaemic control; n = 3 triplicates). (E) Poly-caspase-active cells, (F) remaining attached cell number, (G) mean nuclear area and (H) NFI in response to 5 h sole and combined withdrawal of oxygen, glucose and serum (@P < 0.001 vs all other conditions; *P < 0.05, **P < 0.01 vs corresponding medium in normoxia; ^P < 0.01, ^^P < 0.001 vs full medium in normoxia; #P < 0.01 vs full medium in hypoxia; n = 3 triplicates). (I) Representative images of HL-1 cardiomyocytes deprived of serum and/or glucose in hypoxia, scale bar = 100 μm.
After 5 h of simulated ischaemia, the total number of attached HL-1 cells was significantly higher in the presence of CBMSC-conditioned medium (3.9 ± 0.1 × 10^4 vs 3.3 ± 0.2 × 10^4 in control, \( P = 0.05 \); prior simulated ischaemia: 5.4 ± 0.2 × 10^4) (Fig. 3A). Although the proportion of detached dead cells was significantly decreased in CBMSC-conditioned medium (28 ± 1 vs 39 ± 3% in control, \( P = 0.05 \)), the proportion of poly-caspase-active cells was unchanged when compared with the control (18 ± 3 vs 16 ± 4% in control, \( P = 0.6 \)) (Fig. 3B). In adherent HL-1 cardiomyocytes, late apoptosis, defined by phosphatidylinerse exposure and loss of plasma membrane integrity, was significantly reduced (68 ± 2% of the control, \( P < 0.001 \)), yet necrotic cells were slightly more frequent (1.13 ± 0.04-fold of control, \( P < 0.05 \)) (Fig. 4A). In line with this result, nuclear shrinking, a hallmark of apoptosis, was reduced in CBMSC-conditioned medium (mean nuclear area 108.2 ± 0.8 vs 106 ± 0.8 μm² in control, \( P < 0.05 \)) (Fig. 4B), and post-ischaemic metabolic activity (MTS assay) of HL-1 cardiomyocytes was enhanced in CBMSC-conditioned medium by 12 ± 1% (\( P < 0.05 \) vs control) (Fig. 5A). To determine whether this protective effect is stem cell-specific, we performed the MTS assay with medium conditioned by foreskin fibroblasts and found that metabolic activity after simulated ischaemia was not different (changed by 5 ± 2% vs control, \( P = 1 \)) (Fig. 5A). Finally, we sought to determine whether pro-mitotic activity contributed to the observed beneficial effects. In post-ischaemic HL-1 cardiomyocytes, BrdU uptake was 56 ± 1% of that of non-ischaemic cells (\( P < 0.001 \)), indicating impaired proliferation. BrdU uptake was higher in CBMSC-conditioned medium (70 ± 3%, \( P < 0.001 \) vs control), but unchanged in fibroblast medium (59 ± 2%, \( P = 1 \) vs control) (Fig. 5B).

**Signalling pathway regulation in HL-1 cells during simulated ischaemia**

Akt phosphorylation increased immediately and reached a peak after 4 h, where it was 4-fold higher than under non-ischaemic conditions (Fig. 6A). ERK1/2 phosphorylation decreased during the first hour of simulated ischaemia but then recovered (Fig. 6B) and was 3-fold increased after 5 h. Phosphorylation of STAT3 rapidly declined after the onset of simulated ischaemia to 39% of the baseline value. After 3 h, however, STAT3 phosphorylation rose again and reached 64% of the baseline phosphorylation level after 5 h (Fig. 6C). In the presence of CBMSC-conditioned medium, we detected distinct differences in the phosphorylation pattern of the three survival pathway checkpoints. Measured after 3 h of simulated ischaemia, phosphorylation of Akt and ERK1/2 was enhanced 2- and 3-fold, respectively (\( P < 0.05 \) and 0.01) (Fig. 6D and E), and STAT3 phosphorylation increased >12-fold (\( P < 0.001 \)) (Fig. 6F) compared with control medium. To exclude interference with other STAT protein family members, we also studied the phosphorylation of STAT1 and STAT5 as well as of GSK3β, a downstream target of both Akt and ERK1/2 signalling. Phosphorylation of STAT1 and STAT5 was not detectable in the presence of control medium or CBMSC-conditioned medium (Fig. 6H and I), while GSK3β phosphorylation appeared mildly but not significantly increased (\( P = 0.1 \)) (Fig. 6G). In addition, we also tested the stem cell specificity of the observed effects by subjecting HL-1 cells to simulated ischaemia in the presence of fibroblast-conditioned medium. Here, STAT3 phosphorylation was significantly less pronounced (\( P = 0.37 \) vs control medium, \( P < 0.001 \) vs CBMSC-conditioned medium) (Fig. 6F), while phosphorylation of Akt, ERK1/2 and GSK3β was not significantly different (Fig. 6D, E and G).

**Functional relevance of Akt-, ERK1/2- and STAT3 signalling to cord blood mesenchymal stromal cell-induced protection**

Simulated ischaemia experiments were repeated in the presence of specific small-molecule inhibitors. The efficiency of Wortmannin, UO126 and Statick in suppressing phosphorylation of Akt, ERK1/2 and STAT3 was confirmed by western blot (Fig. 7A-C). Of note, STAT3 inhibition resulted in distinctly increased ERK1/2 phosphorylation, but no further interplay was detected (Fig. 7E). Direct cytotoxic effects of the inhibitors were ruled out by adding them to HL-1 cells in standard culture conditions, where metabolic activity determined by MTS assay remained similar to that of untreated cells (Fig. 7D). In simulated ischaemia, neither individual inhibition of PI3K-dependent Akt phosphorylation nor MEK1/2-dependent ERK1/2 phosphorylation abrogated the beneficial effect of CBMSC-conditioned medium. Blocking STAT3 phosphorylation lowered the viability of ischaemic HL-1 cells in the presence of control medium to 74 ± 5% (\( P < 0.01 \)), but did not influence the beneficial effect of CBMSC-conditioned medium. When Statick was applied in combination with Wortmannin or UO126, viability of
ischaemic HL-1 cells declined in control medium to 69 ± 4 and 70 ± 5%, respectively (both $P < 0.01$), but was not affected in CBMSC medium. The beneficial CBMSC effect was only abolished when both Akt (Wortmannin) and ERK1/2 (UO126) pathways were blocked. Under these conditions, cell viability declined significantly in CBMSC-conditioned medium ($P < 0.05$), but not in control medium ($P = 1$) (Fig. 7F and G).

**DISCUSSION**

Factors secreted by CBMSCs attenuate cell death and activate survival signalling in HL-1 cardiomyocytes subjected to simulated ischaemia in vitro. This effect is, to some extent, specific to CBMSCs and not entirely reproducible by fibroblasts. Akt and ERK1/2 transmit protective signals in the cardiomyocyte cytosol in a compensatory manner, while STAT3, although crucial for cardiomyocyte survival in ischaemia, seems to be no key mediator of the protective paracrine stem cell action.

While the clinical impact of cell therapy in myocardial ischaemia has so far been modest [10–12], non-regenerative cardioprotective effects of stem/progenitor cell products have been demonstrated in several experimental models including H9C2 cells, rat cardiomyocytes, human myocardial tissue culture, isolated hearts and in vivo [3, 5, 13–15]. A better understanding of the molecular mechanisms may ultimately facilitate a more successful clinical translation, and standardized experimental models are required for this purpose. *In vivo* and *in vitro* whole-tissue models are certainly closer to the clinical situation, but do not allow discrimination between events in cardiomyocytes and other cardiac cell types. For our experiments, we chose the HL-1 cell line, established from transgenic mice, in which expression of the SV40 large T-antigen was targeted to atrial cardiomyocytes via the atrial natriuretic factor promoter. These cells contract and retain most of the defining phenotypic characteristics of adult cardiomyocytes [16], but are less costly and easier to handle in cell culture than primary cells or cardiomyocytes derived from embryonic or induced pluripotent stem cells. During ischaemia, supply with oxygen, glucose

![Figure 3: Cell loss and caspase activation in HL-1 cardiomyocytes after simulated ischaemia in cord blood mesenchymal stromal cells-conditioned medium.](image-url)
and humoral constituents is critically deficient or completely interrupted. We therefore developed an in vitro model of combined withdrawal of oxygen, glucose and serum that proved to efficiently induce apoptosis and cell death in our target cells. Many of the studies of cardioprotection conveyed by transplantable cells used heterogeneous cell populations with incompletely defined characteristics and possibly reduced potency, because they were derived from aged and diseased human donors [10–12]. We therefore decided to use human CBMSCs for our experiments, a uniform neonatal cell population with superior proliferative capacity that has not been subjected to ageing and disease.

**Intracellular signalling**

Although reduction in cardiomyocyte apoptosis by MSCs has been reported before, the signalling pathways involved have rarely been investigated [5–7]. In a mouse model of myocardial infarction, transplantation of MSCs was associated with Akt, GSK3β and ERK phosphorylation [17]. Our findings confirm this observation and add information derived from inhibitor experiments. Enhanced phosphorylation of Akt and ERK has been described in HL-1 cells exposed to simulated ischaemia and subsequent reperfusion [18, 19]. Both kinases confer resistance against ischaemia/
reperfusion injury, but require enhancement beyond their endogenous activation to exert protective effects. In our model, only combined blocking of the PI3K/Akt and MEK/ERK signalling abolished CBMSC-mediated cardioprotection. Both pathways converge on common protective endpoints including the inhibition of mitochondrial permeability transition pore opening and suppression of pro-apoptotic Bcl2 family members, and inhibition of one pathway has been shown to activate the other and vice versa [20]. Phosphorylation of STAT3 was enhanced in post-ischaemic isolated mouse cardiomyocytes and in rat hearts [21, 22], and is believed to promote cell survival during ischaemia/reperfusion. In our model, however, STAT3 phosphorylation was clearly suppressed during simulated ischaemia, while further pharmacological inhibition drastically lowered HL-1 cell viability. Interestingly, CBMSC medium dramatically increased STAT3 phosphorylation, and blocking STAT3 in this situation led to ERK activation. Such a crosstalk between STAT3 and ERK has already been described in proximal tubular cells and recently, also in HL-1 cardiomyocytes [23, 24]. However, when STAT3 phosphorylation was blocked alone or in combination with ERK or Akt, the protective CBMSC effect was undisturbed, indicating that STAT3 is not a key mediator of paracrine stem cell actions. Similarly, STAT3 activity alone is not able to counteract the concurrent loss of Akt and ERK signalling.

**Stem cell specificity**

Most investigations compare stem cell effects with cell-free control preparations, and the question whether non-stem cells may exert similar effects is avoided. We found that fibroblast-conditioned medium also activates Akt, ERK and STAT3, albeit to a lesser extent than CBMSC medium, and has indeed been shown to ameliorate survival of cardiomyocytes in hypoxia [25]. Nevertheless, the effects of fibroblast medium on HL-1 cell viability/metabolic activity and proliferation did not match those of

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**Figure 6:** Intracellular signalling pathway regulation in HL-1 cardiomyocytes in response to CBMSC-conditioned medium. Western blots indicating phosphorylation of (A) Akt, (B) ERK1/2 and (C) STAT3 after 1–5 h simulated ischaemia (*P < 0.05, **P < 0.01 vs non-ischaemic control; n = 3). Phosphorylation of (D) Akt, (E) ERK1/2, (F) STAT3 and (G) GSK3β after 3 h simulated ischaemia in fibroblast and CBMSC medium (relative to respective control medium, *P < 0.05, **P < 0.01, ***P < 0.001; n = 9). Phosphorylation of (H) STAT1 and (I) STAT5 in HL-1 cells subjected to 3 h simulated ischaemia in CBMSC medium. Positive control: HeLa cells treated with interferon-α (IFN-α).
CBMSCs in our model, and there were differences regarding the growth factor and cytokine profile of the conditioned media. HGF, a growth factor known for its anti-apoptotic action on cardiomyocytes, was significantly higher in CBMSC medium, in line with our finding of reduced HL-1 cell apoptosis. On the other hand, fibroblast medium contained higher amounts of bFGF and IL-6. The effects of IL-6 on the ischaemic heart are controversial. While it is clearly pro-inflammatory, IL-6 may also provide cardioprotection via the glycoprotein (gp)130/janus kinase (JAK)/STAT axis. However, the weak STAT3 activation we found with fibroblast medium discourages this mechanism in our model.

**Apoptosis vs necrosis**

Overall, CBMSC medium clearly reduced the number of HL-1 cells that had died, detached and lost their plasma membrane integrity. Among the cells that were still attached, CBMSC medium primarily reduced late apoptosis, while poly-caspase activity (early apoptosis) was unchanged and necrosis was even slightly increased. Considering that during the conditioning period CBMSCs may secrete cardioprotective proteins but also reduce the nutrient content of the medium, it appears plausible that signal-regulated apoptotic cell death is suppressed, while more cells ‘passively’ die in necrosis due to diminished energy supply.

**Limitations of the study**

Clearly, the simulated in vitro ischaemia model we used is far removed from the in vivo situation, and inferences regarding the clinical use of cell therapy should be made with caution. HL-1 cells are an immortalized cell line derived from a mouse atrial tumour and are very different from naive ventricular cardiomyocytes. We primarily focused on the cellular response to simulated ischaemia, although several assays also required a reoxygenation period. On the cellular level, reperfusion injury is very different from ischaemia alone, although in the clinical setting they often cannot be clearly distinguished. Cord blood-derived MSCs are also not available for autologous cardiac cell therapy, but, in
principle, they could be applied in an allogenic fashion. Presumably, the cardioprotective activity of MSCs from aged and diseased ‘autologous’ donors is different.

**Conclusion**

A detailed understanding of the molecular mechanisms that underlie cardiac cell therapy is absolutely necessary if one aims at improving its efficacy in the clinical setting. We showed that human CBMSCs exert cardioprotective paracrine effects that are indeed more pronounced than those of fibroblasts. While activation of PI3K/Akt and MEK/ERK signalling both contribute in a mutually compensatory fashion, STAT3 is not involved in mediating the beneficial CBMSC effects. These results provide new insights into the complex signalling network that conveys cytoprotection by factors released from stem cells and may help to better exploit the potential of cell therapy in ischaemic hearts.

**SUPPLEMENTARY MATERIAL**

Supplementary material is available at EJCTS online.

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