Potent endothelial progenitor cell-conditioned media-related anti-apoptotic, cardiotrophic, and pro-angiogenic effects post-myocardial infarction are mediated by insulin-like growth factor-1

Brian Hynes†, Arun H.S. Kumar†, John O'Sullivan, Chirlei Klein Buneker, Anne-Laure Leblond, Sharon Weiss, Jeffrey Schmeckpeper, Kenneth Martin, and Noel M. Caplice*

Centre for Research in Vascular Biology (CRVB), Biosciences Institute, University College Cork, Cork, Ireland

Received 10 June 2011; revised 4 October 2011; accepted 19 October 2011; online publish-ahead-of-print 15 December 2011

Aims
We have previously reported the cardioprotective effects of endothelial progenitor cell (EPC)-conditioned media (CM) therapy post-myocardial infarction (MI). In the present study, we have determined the insulin-like growth factor-1 (IGF-1) contribution to EPC CM effects on cardiomyocyte survival, contractility, and angiogenesis in vivo.

Methods and results
Conditioned media from porcine EPC were administered intracoronary in the presence and absence of specific neutralizing antibodies to IGF-1 or control IgG in a porcine model of MI. X-vivo (non-conditioned) medium was used as a control. Functional, histological, and biochemical parameters were evaluated at 24 h and 8-week post-therapy. Conditioned media therapy significantly abrogated infarct zone (IZ) apoptosis, hypocontractility, and impaired left ventricular (LV) relaxation observed in control infarcts acutely (24 h post-MI). At 8 weeks following treatment, CM therapy augmented LV contractility and relaxation, IZ angiogenesis and inhibited infarct size expansion, wall expansion, and wall thinning. All of these acute and chronic beneficial effects of CM therapy were vitiated by neutralizing antibodies to IGF-1 but not by control IgG. Moreover, the addition of neutralizing IGF-1 antibody to control medium had no effect on these structural or functional changes in the heart post-treatment.

Conclusion
Insulin-like growth factor-1 within the EPC CM mediates potent acute myocardial repair and chronic remodelling effects post-MI. These findings may provide a rationale for comparative trials of specific growth factors vs. current progenitor cell strategies.

Keywords
Porcine endothelial progenitor cell • Conditioned media • Insulin-like growth factor-1 • Myocardial infarction

Introduction
Numerous mechanisms for cell therapy benefit in the context of myocardial infarction (MI) have been proposed including direct and indirect effects of cells to augment ventricular repair, contractile performance, and remodelling.¹–⁶ We and others have shown that factors secreted from culture-modified or genetically manipulated progenitor cells have a range of positive biological effects that may impact left ventricular (LV) repair post-infarction.⁶–¹⁷ Using primary cardiomyocyte cultures exposed to hypoxia, we have previously identified insulin-like growth factor-1 (IGF-1) within the endothelial progenitor cell (EPC) secretome to be cytoprotective at picomolar concentrations.⁶–¹⁷ An obvious question arising from these intriguing studies is: are specific growth factors responsible for these beneficial effects in vivo? Importantly, to date, no direct mechanistic links between specific paracrine factors present at...
picomolar concentrations in progenitor cell-conditioned media (CM) and repair pathways within the myocardium in vivo have been reported in a large animal model of infarction that would approximate the mass of the human heart.

To address this question, we undertook a systematic analysis of selective IGF-1 blockade in the EPC secretome in a porcine model of MI. Here, we provide evidence not only for IGF-1 signalling, but also the potential of IGF-1-related early anti-apoptotic and late positive remodelling, haemodynamic, and pro-angiogenic effects post-MI. These experiments underscore the potency and potential therapeutic value of direct growth factor administration and may provide an impetus for renewed peptide or protein approaches to cardiac repair post-MI.

**Methods**

**Porcine endothelial progenitor cell isolation and generation of endothelial progenitor cell-conditioned media**

Endothelial progenitor cells were generated from 30 to 40 mL of peripheral blood drawn from an ear vein of female Landrace pigs under aseptic technique 48 h before infarct generation, as described previously. Briefly, the mononuclear cell layer isolated by Biocoll gradient centrifugation from peripheral blood was plated on a fibronectin (1 μg/mL)-coated plate in X-vivo-15 medium (Catalogue no: 04-744Q; Lonza) supplemented with vascular endothelial growth factor (1 ng/mL). After 48 h of culture (37°C and 5% CO2), cell-free CM were harvested from the cell culture by centrifugation (600 g for 5 min) and filtration (0.2 μm) of the media. This media were kept on ice and were warmed to room temperature before administration. A 1 mL sample of the CM was collected at 24, 32, and 48 h in culture and analysed for IGF-1 concentration using a commercial ELISA Kit (R&D system).

**Porcine model of myocardial infarction and tissue analysis**

Fifty-six female Landrace pigs (25–30 kg) were used in this study in accordance with the guidelines of the Experimental Animal Ethics Committee of University College Cork. Myocardial infarction was induced as described previously. Briefly, anaesthesia was induced by intramuscular injection of ketamine and xylazine. Mechanical ventilation was carried out using a large animal Harvard Apparatus ventilator and supplemental oxygen (4–6 L/min) combined with isoflurane (1–4%) to maintain general anaesthesia. Percutaneous balloon occlusion of the left anterior descending coronary artery was performed using a large animal Harvard Apparatus ventilator and supplemental oxygen (4–6 L/min) combined with isoflurane (1–4%) to maintain general anaesthesia. Percutaneous balloon occlusion of the left anterior descending coronary artery was performed for 90 min followed by deflation of the balloon and continuous reperfusion for two more hours. At the end of the 2 h reperfusion, three cycles of intracoronary infusion of 4 mL of X-vivo (control), CM, CM + anti-IGF-1 antibody, CM + IgG, or X-vivo + anti-IGF-1 antibody were performed followed by angiography to confirm artery patency (TIMI-3 flow), and the animal was recovered. For the CM + anti-IGF-1, CM + IgG, or X-vivo + anti-IGF-1 group, the blocking antibody or the IgG was mixed in CM 30 min prior to intracoronary delivery.

Twenty-four hours (acute) or 8 weeks (chronic) after infarct generation, animals underwent repeat coronary angiography, haemodynamic parameters were recorded, and then the animals were sacrificed by pentobarbitone overdose. The hearts were explanted, weighed, and sectioned in 5 mm transverse slices from the apex to the base (6–8 slices/heart). Images of the sections were captured using a 10 mega-pixel digital camera. Infarct area, expansion index, and thinning ratio were quantified by planimetry of the images using Image J software (US National Institutes of Health, MD, USA). The myocardium was sampled from infarct border zone and remote myocardial areas for Optimal Cutting Temperature (OCT) embedding and cryopreservation in liquid nitrogen. All analyses were performed by at least two blinded and independent observers.

**Pressure-volume loop protocol**

A 5 Fr pigtail pressure-volume conductance catheter (Miller Instruments, TX, USA) was positioned in the LV apex under fluoroscopic guidance prior to MI generation, post-treatment, and immediately prior to sacrifice. Haemodynamic parameters were recorded under steady state in sinus rhythm for 10 min using LabChart 5 Pro (ADInstruments, Ltd, Oxfordshire, UK) and off-line analysis was performed using PVAN ultra 1.0 software (ADInstruments).

**Histological assessment and staining of infarct zone myocardium**

Five micrometre thick sections of the infarct zone (IZ) myocardium were cut and stained with picrosirius red. Collagen content was quantified as percentage picrosirius red staining of total IZ area in 25 high-powered fields per treatment group using NIS-Elements BR software (Nikon).

**Apoptosis in infarct zone**

The apoptosis signals from the IZ myocardium were quantified by terminal deoxynucleotidyl-mediated dUTP nick-end labelling (TUNEL) (immunofluorescence), protein expression of caspase 9 on western blot, and by measuring caspase 3 and 9 activity. Terminal deoxynucleotidyl-mediated dUTP nick-end labelling-positive signal within cardiomyocytes was validated by co-staining with α-sarcomeric actin. Briefly, at 24 h, the heart was cut into 5 μm thick cross-sections from the apex to the base and stained with 2% TTC stain to delineate the healthy tissue (red) from infarcted area (white). Myocardial tissue was sampled from the infarcted area which was clearly visible as white zone against the reddish-pink healthy tissue. This tissue was cryo-embedded for histological sectioning; 5 μm thick sections were screened under the brightfield view to identify the IZ, which was evident by disrupted muscle fibres. Terminal deoxynucleotidyl-mediated dUTP nick-end labelling-positive cells were counted post-staining (using a commercial kit from R&D systems) in a high-power field (× 40) comprising 50% infarct and 50% normal area.

**Quantification of insulin-like growth factor-1 expression in myocardium**

The infarct border zone myocardial samples at 24 h post-infarction were homogenized and the free and total IGF-1 protein was quantified using commercial free IGF-1 (Beckman-Coulter Diagnostic Systems Laboratories, Inc.) and total IGF-1 ELISA kits (R&D system) as per the manufacturer’s protocol.

**Detection of pro-apoptotic markers and signalling proteins in infarct zone myocardium**

For immunoblot analysis membranes were blocked in 5% milk powder in PBS–Tween 20 (0.05%) before incubation with primary antibodies against caspase 9 (1:100, Stressgen, Brussels, Belgium) or p-AKT (1:200, Santa Cruz Biotechnology Inc., CA, USA), overnight at 4°C. p-Akt blots were stripped and re-probed for total AKT (1:200) also...
applied overnight at 4°C. Membranes were washed and the appropriate secondary peroxidase-conjugated antibody (Jackson ImmunoResearch Laboratories, Inc., PA, USA) was applied for 1 h at room temperature. The bound antibodies were visualized by chemiluminescence (SuperSignal, Pierce). Bands corresponding to the correct molecular weight were quantified using Image J software. Caspase 3 and 9 activity in the IZ was estimated using a commercial kit (Chemicon).

Angiogenesis assessment in the infarct border zone

Immunostaining of CD31 (1:200, PECAM-1; Santa Cruz Biotechnology) was performed and microvessel density (vessels of ≤20 μm in diameter with lumen and possessing CD31 and nuclear staining) was quantified per high-power field employing NIS-Elements BR software.

Statistical analysis

Data are presented as mean ± SEM. One-way analysis of variance was used to carry out multigroup analyses with the Bonferroni post-test analysis to detect differences between groups (two-sided) using GraphPad Prism-v4 (GraphPad Software, Inc., CA, USA). Statistical significance was taken as P < 0.05.

Results

Anti-apoptotic effects of endothelial progenitor cell-conditioned media post-infarction are mediated through insulin-like growth factor-1

Following acute MI, administration of CM after coronary reperfusion significantly reduced the number of TUNEL-positive cells/IZ high-power field when compared with control therapy (Figure 1A and B; P < 0.01). This anti-apoptotic effect of CM in vivo was markedly attenuated by pre-incubation with anti-IGF-1 but not control antibody (Figure 1B; P < 0.01). A sample analysis of IZ co-staining of DAPI, TUNEL, and α-sarcromeric actin showed dual staining of TUNEL with α-sarcromeric actin in 91.7 ± 0.4% of cells examined, suggesting that the majority of apoptotic cells analysed were cardiomyocytes (Figure 1C). Caspase 9 protein expression (0.36 ± 0.06 vs. 1.00 ± 0.10 Ab units, P < 0.01) and activity (6.17 ± 0.30 vs. 15.89 ± 1.35 U/mg protein, P < 0.01) within the IZ were similarly reduced at 24 h post-infarction in CM-treated pigs compared with control pigs (Figure 1D and E). This anti-apoptotic effect of CM was again significantly attenuated by anti-IGF-1 but not control IgG (Figure 1D and E). Similarly, caspase 3 activity was decreased in the CM group compared with control treatment and this effect was completely neutralized by antibodies to IGF-1 but not by IgG isotype (data not shown).

Insulin-like growth factor-1 in porcine endothelial progenitor cell-conditioned media elicits Akt phosphorylation in the infarct zone

To test whether IGF-1 within CM activated specific in vivo signalling pathways within the IZ myocardium, Akt phosphorylation was analysed in this region using western blot analysis. Conditioned media significantly augmented Akt phosphorylation within the IZ compared with control treatment at 24 h post-administration (Figure 2). This augmentation of signalling was completely inhibited by pre-treatment of CM with antibody to IGF-1 (Figure 2). IgG control antibody at similar concentration had no effect on Akt phosphorylation (Figure 2).

Porcine endothelial progenitor cell-conditioned media-induced reduction in left ventricular infarct area at 8-week post-myocardial infarction is inhibited by antibody to insulin-like growth factor-1

Infarct size and creatine phosphokinase levels were approximately the same in all treatment groups at 24 h, suggesting no differences between groups in the extent of initial infarction induced (data not shown). At 8 weeks following treatment, infarct area in animals administered CM was 25.9 ± 2% compared with 39.5 ± 1.9% for the X-vivo-treated control group (Figure 3A; P < 0.01). This 37% reduction in infarct size induced by CM was completely abrogated by addition of neutralizing antibody to IGF-1 but not control antibody (Figure 3A). Additionally, CM therapy compared with the control treatment prevented infarct expansion (0.23 ± 0.05 vs. 0.70 ± 0.08, P < 0.01) and thinning of the infarcted wall (0.50 ± 0.04 vs. 0.28 ± 0.02, P < 0.01) (Figure 3B and C). These positive remodelling effects were again completely abrogated by neutralizing antibody to IGF-1 (P < 0.01 vs. the CM group) but not control antibody (Figure 3B and C). Insulin-like growth factor-1-neutralizing antibody addition to X-vivo medium did not have any additional effects over that observed in the control group (Figure 3A–C).

Insulin-like growth factor-1 in conditioned media increased infarct zone cardiomyocyte cell surface area post-myocardial infarction

A 1.5-fold increase in IZ cardiomyocyte cell surface area was observed in the CM-treated group (Figure 4). However, the number of cardiomyocytes in the IZ was similar in all the groups (data not shown). This hypertrophic effect of CM on IZ cardiomyocytes was abrogated by blocking the IGF-1 in CM using an IGF-1-specific-neutralizing antibody (Figure 4). Neither IgG addition to CM nor neutralizing IGF-1 antibody addition to X-vivo medium had any direct effects on the cardiomyocyte cell surface area (Figure 4).

Conditioned media augmentation of left ventricular function acutely and chronically is mediated by insulin-like growth factor-1

Conditioned media delivery resulted in significant acute improvement in contractility and relaxation (+dp/dt) when compared with control treatment (Figure 5A; P < 0.01) and this improvement in systolic and diastolic function was inhibited by the addition of neutralizing antibody to IGF-1 (Figure 5A; P < 0.01) but not by the addition of control antibody. A similar pattern of improvement in contractility and relaxation indices was observed at 8-week post-treatment (Figure 5B).
Conditioned media-induced increase in infarct border zone angiogenesis at 8-week post-infarction is mediated through insulin-like growth factor-1

Conditioned media exerted a pro-angiogenic effect in the infarct border zone with greater than two-fold increase in microvessels detected by CD31/DAPI dual staining in this region compared with the control treatment (Figure 6). This effect was inhibited by antibody to IGF-1 ($P < 0.01$) but not by control antibody (Figure 6). However, the addition of neutralizing IGF-1 antibody to x-vivo medium had no effect on microvessel density. Analysis of collagen content within the IZ at 8-week post-MI did not reveal any significant differences between the treatment groups, suggesting that levels of fibrosis were not affected (see Supplementary material online, Figure S1).

**Discussion**

Considerable experimental evidence now exists to support a paracrine mechanism for at least part of the beneficial effects of...
progenitor cell therapy post-MI. From a clinical perspective, these studies open the regenerative medicine field up for dissection of specific factors that might be administered in lieu of cells. Indeed, this latter approach may offer several advantages in terms of an ‘off the shelf’ therapy that may be more accessible to the broader clinical community. Likewise, this may be more amenable to dose–response testing, a hallmark heretofore in optimizing pharmacological therapy.

To date, most evidence supporting a paracrine mechanism of cardiac repair derives predominantly from small animal studies with an exception of one study evaluating the cardioprotective effects of thymosin β4 in a porcine model. While the current study identifies IGF-1 as a specific paracrine factor in the EPC secretome, with the goal of developing alternative cardiac repair strategies to current cell therapy approaches in an animal model which in scale, mode, and timing of therapy at least approximates the human acute MI therapeutic scenario. In this study, IGF-1 present in EPC CM exhibited salutary acute and chronic in vivo effects on IZ cardiomyocyte apoptosis, infarct size, angiogenesis, and cardiac function. Indeed, the acute effect on IZ apoptosis may underpin the chronic improvement in infarct size seen at 8-week post-treatment. Thus, chronic beneficial effects on infarct size are likely a reflection of acute gains in cardiomyocyte survival in this region. The anti-apoptotic effects of IGF-1 in CM on cardiomyocytes were not directly evident by infarct size determined by TTC staining in the acute phase owing to insensitivity of TTC to distinguish between cardiomyocytes undergoing apoptosis and those cardiomyocytes protected by IGF-1 therapy. This latter finding is likely due to some TUNEL-positive cardiomyocytes being capable of metabolizing TTC to formazan (data not shown—personal communication AHSK). Importantly, in our study, treatment with CM did not have any significant adverse effect on scar formation as measured by collagen.
content in the IZ. However, the lack of beneficial effects of CM on cardiac fibrosis may be due to co-existence of pro-fibrotic factors (TGF-β-1) in CM which may have countervailing effects to IGF-1.

The acute haemodynamic benefits of CM-derived IGF-1 seen in our study may reflect both an attenuation of acute cardiomyocyte apoptosis and an improvement in cardiac inotropic performance. This latter effect on cardiomyocyte contractility has previously been shown for single growth factor administration in smaller animal and in vitro models. The positive structural and haemodynamic effects seen chronically following CM treatment may also reflect maintenance of the acute gains seen at 24 h. These effects which included increased angiogenesis observed at 8-week post-treatment were consistently neutralized using an antibody to IGF-1 but not control IgG. Moreover, the addition of neutralizing IGF-1 antibody to control X-vivo medium had no structural effects in vivo, suggesting that the benefits of CM were mediated by exogenous IGF-1 contained therein and not by endogenous IGF-1 released at the infarct site.

The cardioprotective effects in our study were more potent than previously reported for IGF-1 delivered alone in animal models of MI. However, differences in time, growth factor concentration, mode of delivery, and infarct model used make direct comparison with previous studies difficult. Interestingly, the concentration range of IGF-1 administered via the intracoronary route in the current study (~600 pg) is at least two orders of magnitude lower in dose than all previous intravenously administered growth factor studies. Moreover, recombinant human IGF-1 used at a similar concentration range to that detected in CM in our in vivo study significantly protected neonatal rat cardiomyocytes in vitro from apoptosis-induced cell death (see Supplementary material online, Figure S2). Uniquely, our data confirms relevant in vivo cardiac signalling through the Akt pathway by IGF-1 in large animal model of MI. We also show that in animals where IGF-1 is neutralized, appropriate inhibition of Akt signalling also occurs with no change in the expression of total or free IGF-1 in the myocardium (see Supplementary material online, Figure S3) at 24 h post-treatment. These data are in contrast to findings by Cho et al. in a rodent model following cell therapy that showed later endogenous growth factor effects beyond 24 h for cells. In the current study, growth factors only were studied, and given the residency of growth factors in vivo and robust effect of

Figure 4 Effect of conditioned media on infarct zone cardiomyocyte cell surface area. Conditioned media therapy (first black bar) increased the surface area of cardiomyocytes in the infarct zone at 8-week post-myocardial infarction. This effect was blocked by neutralizing the insulin-like growth factor-1 in conditioned media (second black bar). High-power (×600) images of infarct zone sections stained with sarcomeric-actin (red), laminin (green), and DAPI (blue) were planimetrized to quantify cardiomyocyte cell surface area. Neither the addition of IgG antibody to conditioned media (third black bar) nor neutralizing insulin-like growth factor-1 antibody to X-vivo medium (fourth black bar) had any effect on cardiomyocyte size. An average of 100–200 infarct zone cardiomyocytes were analysed per pig (n = 4–7/group) and the data are reported as mean ± SEM of 800–1000 cardiomyocytes/group. *P < 0.001 vs. the X-vivo control group (white bar).

Figure 5 Effect of conditioned media on left ventricular function. Systolic and diastolic left ventricular function (+dp/dt) at 24 h (A) and 8-week (B) post-myocardial infarction was significantly improved by conditioned media therapy. The beneficial effects of conditioned media were inhibited by neutralizing antibody to insulin-like growth factor-1 but not by IgG control antibody. Data are expressed as mean ± SEM of 20–25 cardiac cycles/pig (n = 4–7/group). *P < 0.01 vs. conditioned media-treated group.
neutralizing IGF-1 antibody in CM at 24 h, it appears likely that 24 h effects of CM on apoptosis were mediated by exogenous IGF-1. However, we cannot completely exclude a possible therapeutic role for other endogenous cytokines including IGF-1 being up-regulated in vivo at later time points beyond 24 h by a range of growth factors within the conditioned medium. It is also conceivable that conditioned medium including IGF-1 induced recruitment of endogenous progenitors that participated in the cardiac repair response. These mechanistic questions will need to be addressed in future studies.

In addition, the association of signalling inhibition with acute effects on apoptosis reduction, although not definitive evidence of a cause–effect relationship, is strongly suggestive that the acutely positive effects of IGF-1 (even at 600 pg concentration) may be mediated through the Akt signalling. Insulin-like growth factor-1 in CM also significantly attenuated infarct expansion and thinning of the infarcted LV wall, indicating a positive remodelling effect of this cytokine. The co-incidence of improved contractility and decreased volumes and end-diastolic pressure (see Supplementary material online, Figure S4) in the CM-treated group compared with the control group would suggest a haemodynamically adaptive response. Importantly, no significant maladaptive hypertrophy was seen at sites remote from infarction (data not shown). This may not be surprising given the concentrations of IGF-1 administered and the targeted mode of delivery.

In conclusion, this study suggests that picogram concentrations of IGF-1 administered in CM via the intracoronary route have potent acute and chronic beneficial effects on cardiac repair post-MI. The causal relationship between specific growth factor-mediated signalling and improvements in apoptosis, ventricular relaxation, contractility, remodelling, and angiogenesis remains to be formally proven. However, based on the current doses administered, further receptor signalling and mechanistic studies, using intracoronary administration of a single purified growth factor, are warranted.

Supplementary material

Supplementary material is available at European Heart Journal online.

Acknowledgements

We thank Barbara Rejman-Bochenek, Scott McCauley, and Graine Murphy for technical assistance with the studies. Health Research Board, Ireland (N.M.C. and B.H.).

Funding

This work was supported by grants from Science Foundation Ireland, Dublin, Ireland (R11482 and RP06 to N.M.C.), Irish Heart Foundation, Dublin, Ireland (R12348 to B.H. and A.H.S.K.), and Molecular Medicine Ireland, Dublin, Ireland (R12699 to J.O.S.).

Conflict of interest: none declared.

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