

Unexpected calcification after direct intravascular injection of autologous bone marrow-derived cells in a chronic total occlusion model

Nafiseh Nili^{1,2}, PhD; Sang Yup Lim^{1,3}, MD, PhD; Beiping Qiang^{1,2}, MD, PhD; Xiuling Qi^{1,2}, MD, PhD; Max J. Weisbrod¹, BSc; Michelle Ladouceur-Wodzak^{1,2}, RLATG; Naomi Gofine¹, BA; Thomas A. Berk¹, BSc; Jamie Saperia¹, BSc; Graham A. Wright^{1,2}, PhD; Bradley H. Strauss^{1*}, MD, PhD

1. Schulich Heart Centre, Sunnybrook Health Sciences Centre, Toronto, ON, Canada; 2. Department of Medical Biophysics, Sunnybrook Health Sciences Centre, University of Toronto, Toronto, ON, Canada; 3. Korea University Ansan Hospital, Ansan, South Korea

N. Nili and S.Y. Lim contributed equally to this manuscript.

KEYWORDS

- angiogenesis
- calcification
- chronic total occlusions
- stem cell therapy

Abstract

Aims: Patients with symptomatic chronic total occlusions (CTO) remain a therapeutic challenge. Enhancement of intraluminal neovascularisation by pro-angiogenic therapies has been proposed as a new strategy to improve percutaneous revascularisation. The aim of this study was to investigate the effects of intraluminal injection of bone marrow-derived cells (BMC) into experimental CTO.

Methods and results: CTO were created in the femoral arteries of 43 New Zealand White rabbits using the thrombin injection model. At 12 weeks following CTO creation, 33 rabbits were injected with either cultured BMC (n=19) or control DMEM alone (n=14) directly into the CTO. Ten rabbits were used for cell tracking (seven BMC and three control). BMC labelled with fluorescent Qdot[®] nanocrystals were identified in the CTO up to one week after injection. Animals were sacrificed at three to five weeks post-treatment and arterial samples were excised for micro-CT imaging and histologic morphometric analysis. There was a significant but modest increase in neovascularisation in BMC-treated arteries compared to controls (7.47±4.75% vs. 4.35±2.97%, p<0.05). However, unexpected intravascular calcification was only detected within the CTO in BMC cell treated arteries. Western blot for conditioned medium from BMC showed up-regulation of osteogenic proteins (BMP-2 and -7).

Conclusions: Although direct delivery of BMC into CTO increases neovascularisation, undesirable vascular calcification will limit this therapeutic approach.

*Corresponding author: Schulich Heart Centre, Sunnybrook Health Sciences Centre, 2075 Bayview Avenue, D4-06, Toronto, Ontario, M4N 3M5, Canada. E-mail: bradley.strauss@sunnybrook.ca

Introduction

Chronic total occlusions (CTO) are reported in approximately 20% of patients with coronary artery disease who undergo coronary angiography. Despite significant symptoms in a large number of these patients, percutaneous coronary intervention (PCI) is attempted in a small proportion of CTO patients¹. The reluctance is frequently due to an anticipated procedure failure related to unsuccessful guidewire crossing. Previous studies by our group have indicated an intricate maturation process within an occluded artery that begins as a thrombus-laden channel in the initial phase, and then progresses to a proteoglycan-rich matrix with abundant microvessels at six weeks, and later to a collagen-rich, poorly vascularised matrix at three months and beyond². Therapeutic angiogenesis achieved by local delivery of an angiogenic agent has been raised as a treatment option to facilitate guidewire crossing, either by providing small channels for the guidewire to traverse the CTO or by softening the occlusion and altering tissue compliance³⁻⁶. We previously reported that local delivery of VEGF¹⁶⁴ protein into CTO increased microvessel formation and facilitated guidewire crossing across the proximal fibrous cap⁷. In the current study, we hypothesised that an alternative angiogenic approach, injection of bone marrow-derived cells (BMC) directly into CTO, would result in an even more enhanced intravascular neovascular response. Thus, the aim of this study was to investigate the effects of intraluminal injection of BMC in an experimental CTO model.

Materials and methods

IN VITRO

BONE MARROW PREPARATION AND CELL CULTURE

Bone marrow (3 to 5 ml) was aspirated from rabbit iliac crest and collected in heparin blood collection tubes containing 2 ml of DMEM (Invitrogen, Life Technologies, Grand Island, NY, USA) supplemented with antibiotics and 10% fetal bovine serum (FBS). Bone marrow samples were filtered through a pre-wet 70- μ m cell strainer and the filtrate was centrifuged (400 \times g, 5 min, 22°C). The pellets containing BMC were washed four times with PBS containing antibiotics and 2% FBS. To get a sufficient cell number and support cell differentiation toward the endothelial cell lineage, cells were cultured on either fibronectin-coated culture dishes (Cat# CACB-356009; BD Biosciences, Franklin Lakes, NJ, USA) according to a modification of Iwase et al⁸, or on cover slides, in endothelial growth medium-2 microvascular (EGM-2MV; Cat# CC-4147; Lonza, Walkersville, MD, USA). EGM-2MV consists of endothelial basal medium-2, 5% fetal bovine serum (FBS), and supplemental growth factors, vascular endothelial growth factor (VEGF), basic fibroblast growth factor, epidermal growth factor, and insulin-like growth factor-1. After four days, non-adherent haematopoietic cells were removed and fresh EGM-2 was added to the adherent BMC.

For CTO injections, cell layers grown for seven days were dissociated from culture dishes using StemPro[®] Accutase[®] (Cat# A11105-01; Invitrogen, Life Technologies), washed three times with ice-cold PBS and suspended in 500 μ l of DMEM without supplements. Cells were kept on ice until injection.

CHARACTERISATION OF BMC

Characterisation of BMC cultured on fibronectin was done on day seven, the time point at which the cells were injected into CTO. For immunostaining, after paraformaldehyde fixation, cells were incubated with 1:10 dilution of monoclonal antibodies CD34 (Cat# M1636; Sanquin, Amsterdam, The Netherlands), CD31 (Cat# JC70A; Dako, Glostrup, Denmark), CD146 (Cat# MAB16985H; Millipore, Billerica, MA, USA), CD14 (Cat# MRB1430; Antigenix America Inc., Melville, NY, USA), RAM-11 (Cat# M0633; Dako), CD45 (Cat# MRB4520; Antigenix), 1:50 dilution of eNOS and caveolin-1 (Cat# 610057; BD Transduction Laboratories, Franklin Lakes, NJ, USA), 1:40 dilution of vitronectin receptor, and 1:200 dilution of osteonectin (Cat# M125; Takara Bio Inc., Otsu, Shiga, Japan) at 4°C, overnight. Slides were incubated with 1:50 dilution of Alexa Fluor 488 rabbit anti-mouse IgG (Cat# A11059; Invitrogen, Life Technologies), at room temperature for one hour. All stains were counterstained with DAPI (1 μ g/ml) and mounted with fluorescent mounting media (Cat# S3023; Dako). Functional characteristics of cultured BMC were assessed by acLDL uptake Dil-acLDL (molecular probes), and *in vitro* angiogenesis using Matrigel (Millipore), according to the manufacturer's protocol⁹. Flow cytometric analysis was also performed to detect the expression of CD31, CD34 and CD146 as markers for endothelial cell lineage.

WESTERN BLOT ANALYSIS OF CONDITIONED MEDIUM

To identify angiogenic and osteogenic proteins, western blotting was performed. After seven days in culture, cells were washed three times with PBS and incubated in serum-free basal medium (EBM-2 Clonetics; Cat# CC-3156; Lonza) for four days. Conditioned medium was collected, mixed with an equal volume of 20% TCA, and kept at 4°C overnight to precipitate proteins. The precipitate was washed three times with 100% ethanol and dissolved in warm (65°C) SDS sample loading buffer. Aliquots corresponding to 15 μ g of cell protein were analysed by 4-20% SDS-PAGE and electrotransferred onto nitrocellulose membranes. Membranes were immunoblotted with rabbit anti BMP-2 (Cat# ARP45066; Aviva Systems Biology Corp., San Diego, CA, USA) and rabbit anti BMP-7 (Cat# AV32329; Sigma-Aldrich, St. Louis, MO, USA) antibodies. Anti rabbit IgG-HRP secondary antibody (Cat# SC-2313; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used for detection of primary antibodies and revealed using chemiluminescence detection system followed by autoradiography using BioMax film.

MINERALISATION ASSAY

To evaluate calcium deposits by BMC in one-week and two-week cultures, Alizarin Red S (ARS) staining was performed¹⁰.

IN VIVO CTO MODEL

The *in vivo* study protocol was approved by the Animal Care Committee at Sunnybrook Health Sciences Centre. Arterial occlusions were created in 43 male New Zealand White rabbits (Charles River Canada, St. Constant, Quebec, Canada) fed a normal rabbit diet, by injection of thrombin solution (200 IU; Cat# 82-036-3; Millipore, Kankakee, IL, USA) into a femoral artery segment, as previously described⁷. The distal ligature was maintained for 60 minutes to ensure a persistent occlusion.

IN VIVO STUDIES

At 12 weeks following CTO creation, 33 rabbits were anaesthetised and injected with either the unselected cultured BMC (n=19) or DMEM media alone (n=14) directly into the CTO.

Rabbits were treated with heparin (1,200 USP units; Pharmaceutical Partners of Canada Inc., Richmond Hill, ON, Canada). A 5 Fr MB1 guiding catheter (Medtronic Inc., Minneapolis, MN, USA) was advanced into the distal abdominal aorta through a 5 Fr sheath placed in the right common carotid artery. Under fluoroscopic guidance, a FineCross microcatheter (Terumo, Somerset, NJ, USA) was positioned directly in contact with the proximal part of the CTO. After removal of the guidewire, BMC (median 3.3×10^6 , 25th percentile 1.3×10^6 ; 75th percentile 4.5×10^6) in 500 μ l DMEM media were delivered through a FineCross microcatheter (Terumo). The injection was done slowly over three to five minutes. The microcatheter was then flushed with an additional 500 μ l of DMEM medium, and left in place for 30-40 minutes prior to removal. The rabbits were recovered and returned to the cage for an additional three to five weeks (i.e., 15-17 weeks since the CTO was created). Since there were no significant intergroup differences between three-week (n=21) and five-week (n=12) groups, the results were pooled. All of the animals had CTO confirmation by angiography. Following sacrifice, CTO specimens were collected, fixed in 10% neutral buffered formalin, embedded in paraffin, stained with haematoxylin/eosin, and the samples were examined under light microscopy.

In a separate series of experiments to track injected cells within the CTOs, BMC were labelled with red-fluorescent Qdot[®] 655 nanocrystals (Cat# Q25021MP; Invitrogen, Life Technologies) before injection, according to the manufacturer's protocol (n=7). Control CTOs were injected with DMEM medium (n=3). CTO injections were performed as described above. We injected labelled BMC to seven CTO rabbits and sacrificed them at four hours (n=3), 24 hours (n=1) and one week (n=3) after cell injection. Control CTO rabbits were sacrificed at four hours (n=2) and at one week (n=1) after cell injection. CTO specimens were embedded in OCT, snap frozen in liquid nitrogen and stored at -80°C until use. Samples were then examined under fluorescence microscopy for labelled cells.

MICRO-COMPUTED TOMOGRAPHY

Micro-computed tomography was performed on excised arteries in an *ex vivo* set-up, as previously described^{7,11}. Following sacrifice, a 5 Fr arterial sheath was positioned in the abdominal aorta. After flushing with normal saline, Microfil[®] (Flowtech Inc., Carver, MA, USA) was injected to fill the vascular structures and form an elastomeric gel, creating a three-dimensional cast of the vasculature within 60 minutes. Femoral arteries were excised within the surrounding muscles and specimens were fixed in 10% neutral buffered formalin. Specimens were embedded in 4% of Difco[™] Agar (BD Diagnostics, Franklin Lakes, NJ, USA) and placed into 40 ml clear plastic tubes. This preparation was scanned using GE Healthcare eXplore Locus SP (GE Healthcare, London, ON, Canada) at a spatial resolution of 14 μ m. A 3D volume image was

reconstructed from the stack of x-ray microtomographic cross-sections normal to the axis of rotation using the GE MicroView software package. Image post-processing and 3D volume rendering comprised several sequential steps. A 3D volume image was reviewed in the GE MicroView software to define the region of interest with subsequent conversion into PNG format by using the MRicro software package (University of South Carolina, Columbia, SC, USA). The 3D volume rendering and image quantification were performed in Amira[®] 3D visualisation software for Windows[®] (Visage Imaging Inc., Andover, MA, USA).

The relative blood volume index (RBVI) in micro-CT was determined as previously described^{7,11}. Specifically, RBV for micro-CT is the ratio of signal intensity from an ROI in the proximal part of the CTO relative to that from an ROI in a patent vessel immediately adjacent to the CTO.

HISTOLOGY ANALYSIS

The arterial specimens were cut into five segments, each of 3-4 mm length. In paraffin embedded specimens, 5 μ m-thick cross-sections of the femoral artery were stained with haematoxylin/eosin and Movat's pentachrome, and Von Kossa stains. Serial slides were analysed to determine the most proximal segment of the CTO to identify the origin of the CTO and the location of the proximal fibrous cap. Morphometric analysis was performed in the most proximal section of the CTO to determine the overall size of the artery (defined by the external elastic lamina), the number of microchannels per section, and the total microvessel area within the vessel, i.e., the combined medial and intraluminal microvessel area as defined by the border of the elastic lamina externa, as previously described⁷. The percent of microvascular area was calculated as the percent of total microvessel area relative to the overall vessel size. Calcification was assessed in Von Kossa stained cross-sections.

STATISTICS

Data are presented as continuous variables and expressed as mean \pm SD. Categorical variables data are expressed in frequencies (%). For comparisons of the data, analyses of variance (ANOVA) with a Student's t-test were performed where the F-test among groups was statistically significant. Statistical analysis was done with the Statistical Package for Social Science (SPSS) software version 17.0 for Windows (SPSS Inc., Chicago, IL, USA). A p-value of <0.05 was considered statistically significant.

Results

IN VITRO RESULTS

CHARACTERISATION OF BONE MARROW-DERIVED CELLS

Cells cultured on fibronectin for seven days were examined. Light microscopy examination showed that the BMC were morphologically heterogeneous and consisted of round cells and spindle-shaped cells and formed cord-like and luminal structures. At the end of the first week, or beginning of the second week, dotted cells appeared and their population increased with time. At one month, circular structures appeared.

Fluorescence microscopy showed that the cultured BMC expressed CD34 (**Figure 1A**), CD31 (**Figure 1B**), CD146 (**Figure 1C**) and eNOS (**Figure 1D**), all markers of endothelial lineage cells. Moreover, the cells expressed caveolin-1, a protein known to associate with eNOS in mature ECs (**Figure 1E**). BMC took up acLDL (**Figure 1F**) and rapidly formed network structures in Matrigel in four hours (**Figure 1G**), indicating functional characteristics of ECs. In addition, some cells stained positive for CD14, a monocyte marker (**Figure 1H**), and for RAM-11, a macrophage marker (**Figure 1I**). Flow cytometric analysis showed positive staining for CD31 (0.55%), CD34 (8.31%), CD146 (11.4%), CD45 (1.04%) and vitronectin receptor (20.5%).

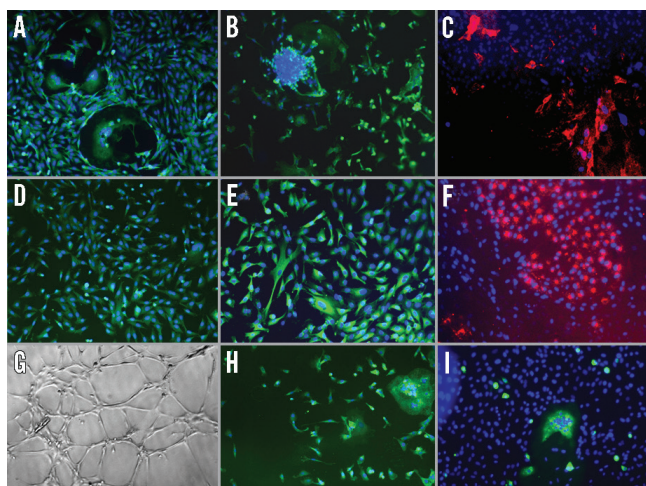


Figure 1. Characterisation of cultured bone marrow-derived cell (BMC) at one week, the time point at which cells were injected into CTO. A) Cells stained positive for CD34 (EPC marker). B) CD31 (EC marker). C) CD146 (red, EC marker). D) eNOS (EC marker). E) Caveolin-1 (EC marker). F) Cells took up acLDL (red), demonstrating functional characteristics of EC. G) Cells formed capillary networks in Matrigel demonstrating functional characteristics of EC. H) Cells stained positive for CD14 (monocyte marker). I) RAM-11 (green, macrophage marker). EC: endothelial cell; EPC: endothelial progenitor cell

IN VIVO STUDIES

BMC TRACKING STUDY

Red-fluorescent Qdot® 655 nanocrystal labelled BMC were identified by fluorescence microscopy within all the CTO at the three time points (four hours, 24 hours and one week) (**Figure 2**). Control arteries injected with medium alone were negative for Qdot nanocrystal immunofluorescence, indicating the specificity of the method. These studies indicate that the BMC were identifiable within the CTO for at least one week following the injection of the cells.

MICRO-CT ANGIOGRAPHY AND HISTOLOGY

There was a significant increase in neovascularisation within the occluded lumen in cell-treated CTOs as determined by both micro-CT and histology (**Figure 3**). The blood volume index determined by micro-CT showed a significant increase in BMC-treated arteries

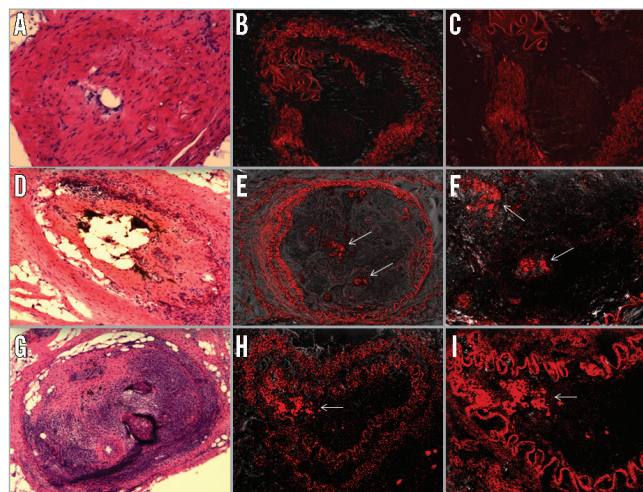


Figure 2. Tracking of BMC into CTO using Qdot nanocrystals to confirm delivery and residency of BMC. The cells were labelled with Qdot® 655 nanocrystal and injected into CTO. Rabbits were sacrificed at four hours and one week and the samples were analysed by fluorescence microscopy. A)–C) Control-Medium. H & E staining (A). Qtracker labelled cells ($\times 10$) (B). Qtracker labelled cells ($\times 20$) (C). D)–F) Labelled cells 4 hours after injection. H & E staining (D). Labelled cells ($\times 10$) (E). Qtracker labelled cells ($\times 20$) (F). G)–I) Labelled cells 1 week after injection. H & E staining (G). Labelled cells ($\times 10$) (H). Labelled cells ($\times 20$) (I). White arrows indicate the cells labelled with Qdot® 655 nanocrystal (red).

compared to controls ($7.47 \pm 4.75\%$ vs. $4.35 \pm 2.97\%$, $p < 0.05$). However, an unexpected finding was the presence of calcification within the CTO in seven of 19 (37%) BMC-treated arteries (**Figure 4A** and **Figure 4B**). No calcifications were evident in any of the control arteries.

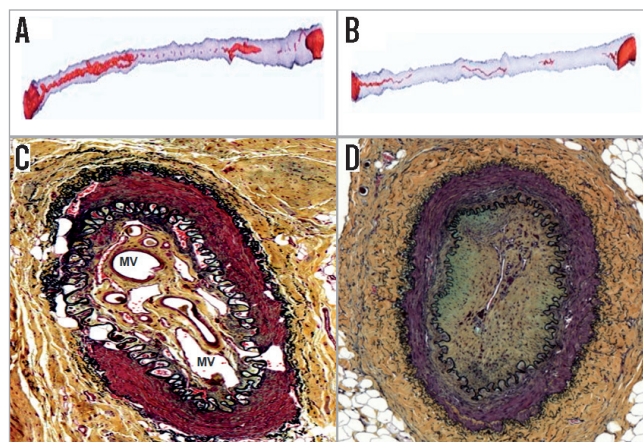


Figure 3. Volumetric images obtained from micro-CT analysis show increased intraluminal neovascularisation in BMC-treated CTO which was confirmed by histology results. A) Micro-CT analysis of BMC-treated CTO. B) Micro-CT analysis of control group. Histology showed increased microvessels in BMC-treated CTO. C) Histology of BMC-treated CTO. D) Histology of control group. MV: microvessel

formation⁷, we hypothesised that local injection of BMC directly into CTO would alter the architecture by increasing neovascularisation. Previous studies from our group have suggested that increasing CTO intraluminal neovascularisation may enhance guidewire crossing⁷. To obtain adequate cell numbers for cell delivery, we cultured cells obtained by bone marrow aspiration. The cultured BMC showed surface markers of endothelial lineage cells by both fluorescence microscopy and flow cytometric analysis. Also, the cultured BMC demonstrated functional characteristics of endothelial lineage cells by taking up acLDL and rapidly formed network structures in Matrigel.

Since this was the first study of BMC effects in CTO, it was not known whether the BMC could penetrate into the CTO and still be evident in the early period after injection. We were reassured by the labelled BMC studies that the cells could be readily identified in the body of the CTO at least one week after the injection. In this model, it is extremely challenging to determine the duration and absolute number of BMC present at different levels of the CTO. Tracking of labelled BMC with red-fluorescent Qdot[®] 655 nanocrystals indicated that the BMC were localised to the proximal and the mid section of the CTO. The presence of the cells, even at one week post injection, and the biologic effects (angiogenesis and calcification) of the cells suggest that these BMC were functional and active. We consider it highly unlikely that a significant number of BMC entered collaterals (which are typically outside the vessel wall) and re-entered the systemic circulation since we performed a direct injection into the CTO.

Similarly, the optimal cell number for delivery and therapeutic effect is not known. Given the limited amount of bone marrow that we could aspirate, it was necessary to culture the BMC to expand the cell population. After seven days of culture, the cell numbers isolated for injection had some variability (1.3–4.5×10⁶, 25th–75th percentile), but the adequacy of the cell number was supported by a significant increase in both percent blood volume measured by micro-CT and by total microvascular area measured by histology. The increase in blood volume index in the BMC-treated group as determined by micro-CT was similar to our previous study of VEGF protein injection in the same model⁷.

Despite the positive results of this approach (penetration of the BMC into the body of the CTO and the modest increase in angiogenic response), we also observed significant intravascular calcification. The calcification would probably be clinically significant, based on its overall extent and its location within the occluded lumen. The presence of calcification is a recognised risk factor for failure of PCI revascularisation¹⁷. In the current study, there was no correlation between delivered cell number and microvessel area or ossified area. The reasons for the absence of a relationship between cell number and these effects is unknown, but may be related to the relatively narrow range of cell number injected. Alternatively, these effects may be due to a particular subset of cells which was not directly quantified.

The mechanisms by which transferred BMC modulate vascular calcification remain unclear. There was no significant correlation between delivered cell number and ossified area. The first possible

mechanism is the influence of the cellular origins of BMC that we delivered. Bone marrow is composed of a mixture of haematopoietic progenitor cells, mesenchymal cells, adipocytes, fibroblastic cells, and osteoblasts¹⁸. It has been well established that mesenchymal cells can differentiate into osteoblasts, chondrocytes, adipocytes, vascular cells or cardiomyocytes under special conditions^{19–22}. Although our cultured cells showed endothelial cell lineage markers such as CD31, CD34, CD146, eNos and caveolin-1, other stem cells could be mixed and promote vascular calcification. It is not clear whether endothelial progenitor cell lineage or other mesenchymal stem cells are involved and responsible for calcification in this CTO model.

Second, the possible interaction between the transplanted BMC and the resident vascular cells in the arterial wall cannot be excluded. The outcome of transplanted cells is likely to be influenced by the method of cell isolation, dose, timing of transplantation and clinical setting. There is a possibility that unwanted differentiation may occur after BMC transplantation in a situation prone to be calcified such as our CTO model. In our CTO model, the osteoclast-osteoblast interaction mediated via bone morphogenetic protein (BMP) may be a potent contributing mechanism of vascular calcification. In our *in vitro* studies, one-week cell cultures were positive for osteoclast markers but negative for osteoblast markers, and there was no evidence of mineralisation. However, at two weeks osteoclast markers were no longer identified but both osteoblast markers and mineralisation were present. Activated osteoclasts produce BMPs, growth factors such as insulin-like growth factor-1 (IGF-1) and transforming growth factor-beta (TGF-β): these factors might stimulate differentiation and activation of osteoblasts^{23,24}. In addition to this osteoclast-osteoblast interaction, BMP-2 and BMP-7 may promote vascular calcification via increased phosphate uptake and induction of osteogenic phenotype modulation in smooth muscle cells^{25,26}, and by promoting proliferation and differentiation of undifferentiated BMC to cartilage and bone²⁷. The relationship between BMC-mediated calcification and interactions with osteoclasts, osteoblasts and BMP merit further investigation.

Indeed, concerns have been raised previously about the potential adverse effects of stem cell transplantation *in vivo*^{28–30}. Injection of mononuclear cells isolated from mouse bone marrow into perinfarct zones of myocardium has resulted in the development of malignant sarcomas²⁸. Intramyocardial calcification has been reported following injection of unselected bone marrow stem cells in mice²⁹. Recently, Liao and colleagues reported vascular calcification in the media of rat aortic arteries at six weeks following balloon injury and injection with bone marrow-derived mesenchymal stem cells³⁰. Our results provide another cautionary example of the potential toxicity of therapeutic applications of cell transplantation in the vasculature.

Study limitations

Since stem/stromal cell lines and stem cell lines are not identical, their characterisation and function can vary depending on their origin and isolation method. Further investigation is required to determine whether one should use unstimulated bone marrow-derived

cells or a more differentiated cell line for neovascularisation or regeneration of a tissue. Our results cannot be generalised to other specific cell lines.

A potential concern of this therapeutic approach is that neovascularisation within the CTO could promote plaque destabilisation. Preclinical and clinical human coronary studies have identified plaque neovascularisation as an important factor in the generation of vulnerable plaque leading to acute myocardial infarction³¹. It is not known whether this could also occur in chronic total occlusions which have been successfully revascularised. Thus, the long-term effects of inducing an angiogenic response on a chronic total occlusion, such as predisposing to plaque rupture and acute coronary syndromes or to stent thrombosis, merits further study.

Conclusion

In summary, although cultured BMC expressed cell surface markers of angiogenic cell types, secreted angiogenic proteins into conditioned medium and showed significantly increased *in vivo* neovascularisation response, injection of these BMC was complicated by the appearance of undesirable extracellular calcifications in an experimental CTO model. This study indicates that caution should be exercised when using cultured BMC for angiogenic effects in CTO.

Impact on daily practice

Chronic total occlusions (CTO) remain a complex group of lesions to revascularise by PCI. Intraluminal microvessels form during early stages of CTO formation and may facilitate PCI, but regress over time as the CTO matures. A strategy to increase intraluminal neovascularisation by injection of bone marrow-derived cells with angiogenic markers into mature CTO resulted in a modest increase in microvessels, but unwanted intraluminal calcification limits the potential clinical utility of this therapeutic strategy.

Funding

Supported by the Canadian Institute of Health Research (grant number MOP - 53325; grant number CTP 82943).

Conflict of interest statement

B.H. Strauss holds a patent for the use of angiogenic therapy, including bone marrow-derived cells, in chronic total occlusions. The other authors have no conflicts of interest to declare.

References

1. Fefer P, Knudtson ML, Cheema AN, Galbraith PD, Osherov AB, Yalonetsky S, Gannot S, Samuel M, Weisbrod M, Bierstone D, Sparkes JD, Wright GA, Strauss BH. Current perspectives on coronary chronic total occlusions: the Canadian Multicenter Chronic Total Occlusions Registry. *J Am Coll Cardiol*. 2012;59:991-7.
2. Jaffe R, Leung G, Munce NR, Thind AS, Leong-Poi H, Anderson KJ, Trogadis J, Nadler A, Shiff D, Saperia J, Lockwood J,

Jacobs C, Qiang B, Teitelbaum A, Dick AJ, Sparkes JD, Butany J, Wright GA, Strauss BH. Natural history of experimental arterial chronic total occlusions. *J Am Coll Cardiol*. 2009;53:1148-58.

3. Strauss BH, Goldman L, Nili N, Butany J, Jackson ZS, Segev A, Eskandarian MR, Sparkes J, Virmani R. Collagenase plaque digestion for facilitating guidewire crossing in chronic total arterial occlusions. *Circulation*. 2003;108:1259-62.

4. Strauss BH, Osherov AB, Radhakrishnan S, Mancini GB, Manners A, Sparkes JD, Chisholm RJ. Collagenase Total Occlusion-1 (CTO-1) trial: a phase I, dose-escalation, safety study. *Circulation*. 2012;125:522-8.

5. Fefer P, Carlino M, Strauss BH. Intraplaque therapies for facilitating percutaneous recanalization of chronic total occlusions. *Can J Cardiol*. 2010;26 Suppl A:32A-36A.

6. Strauss BH, Segev A, Wright GA, Qiang B, Munce N, Anderson KJ, Leung G, Dick AJ, Virmani R, Butany J. Microvessels in chronic total occlusions: pathways for successful guidewire crossing? *J Interv Cardiol*. 2005;18:425-36.

7. Teitelbaum AA, Qi X, Osherov AB, Fraser AR, Ladouceur-Wodzak M, Munce N, Qiang B, Weisbrod M, Bierstone D, Erlich I, Sparkes JD, Wright GA, Strauss BH. Therapeutic angiogenesis with VEGF¹⁶⁴ for facilitation of guidewire crossing in experimental arterial chronic total occlusions. *EuroIntervention*. 2013;8:1081-9.

8. Iwase T, Nagaya N, Fujii T, Itoh T, Murakami S, Matsumoto T, Kangawa K, Kitamura S. Comparison of angiogenic potency between mesenchymal stem cells and mononuclear cells in a rat model of hindlimb ischemia. *Cardiovasc Res*. 2005;66:543-51.

9. Kamihata H, Matsubara H, Nishiue T, Fujiyama S, Tsutsumi Y, Ozono R, Masaki H, Mori Y, Iba O, Tateishi E, Kosaki A, Shintani S, Murohara T, Imaizumi T, Iwasaka T. Implantation of bone marrow mononuclear cells into ischemic myocardium enhances collateral perfusion and regional function via side supply of angioblasts, angiogenic ligands, and cytokines. *Circulation*. 2001;104:1046-52.

10. Cai L, Johnstone BH, Cook TG, Tan J, Fishbein MC, Chen PS, March KL. IFATS collection: human adipose tissue-derived stem cells induce angiogenesis and nerve sprouting following myocardial infarction, in conjunction with potent preservation of cardiac function. *Stem Cells*. 2009;27:230-7.

11. Munce NR, Strauss BH, Qi X, Weisbrod MJ, Anderson KJ, Leung G, Sparkes JD, Lockwood J, Jaffe R, Butany J, Teitelbaum AA, Qiang B, Dick AJ, Wright GA. Intravascular and extravascular microvessel formation in chronic total occlusions a micro-CT imaging study. *JACC Cardiovasc Imaging*. 2010;3:797-805.

12. Lunde K, Solheim S, Aakhus S, Arnesen H, Abdelnoor M, Egeland T, Endresen K, Ilebekk A, Mangschau A, Fjeld JG, Smith HJ, Taraldsrud E, Groggaard HK, Bjornerheim R, Brekke M, Muller C, Hopp E, Ragnarsson A, Brinchmann JE, Forfang K. Intracoronary injection of mononuclear bone marrow cells in acute myocardial infarction. *N Engl J Med*. 2006;355:1199-209.

13. Dimmeler S, Burchfield J, Zeiher AM. Cell-based therapy of myocardial infarction. *Arterioscler Thromb Vasc Biol*. 2008;28:208-16.

14. Lipinski MJ, Biondi-Zoccai GG, Abbate A, Khianey R, Sheiban I, Bartunek J, Vanderheyden M, Kim HS, Kang HJ, Strauer BE, Vetrovec GW. Impact of intracoronary cell therapy on left ventricular function in the setting of acute myocardial infarction: a collaborative systematic review and meta-analysis of controlled clinical trials. *J Am Coll Cardiol.* 2007;50:1761-7.
15. Lian Q, Zhang Y, Zhang J, Zhang HK, Wu X, Zhang Y, Lam FF, Kang S, Xia JC, Lai WH, Au KW, Chow YY, Siu CW, Lee CN, Tse HF. Functional mesenchymal stem cells derived from human induced pluripotent stem cells attenuate limb ischemia in mice. *Circulation.* 2010;121:1113-23.
16. Kinnaird T, Stabile E, Burnett MS, Epstein SE. Bone marrow-derived cells for enhancing collateral development. *Circ Res.* 2004;95:354-63.
17. Hsu JT, Kyo E, Chu CM, Tsuji T, Watanabe S. Impact of calcification length ratio on the intervention for chronic total occlusions. *Int J Cardiol.* 2011;150:135-41.
18. Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science.* 1997;276:71-4.
19. Alhadlaq A, Mao JJ. Mesenchymal stem cells: isolation and therapeutics. *Stem Cells Dev.* 2004;13:436-48.
20. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. *Science.* 1999;284:143-7.
21. Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM. Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature.* 2002;418:41-9.
22. Makino S, Fukuda K, Miyoshi S, Konishi F, Kodama H, Pan J, Sano M, Takahashi T, Hori S, Abe H, Hata J, Umezawa A, Ogawa S. Cardiomyocytes can be generated from marrow stromal cells in vitro. *J Clin Invest.* 1999;103:697-705.
23. Garimella R, Tague SE, Zhang J, Belibi F, Nahar N, Sun BH, Insogna K, Wang J, Anderson HC. Expression and synthesis of bone morphogenetic proteins by osteoclasts: a possible path to anabolic bone remodeling. *J Histochem Cytochem.* 2008;56:569-77.
24. Komarova SV, Smith RJ, Dixon SJ, Sims SM, Wahl LM. Mathematical model predicts a critical role for osteoclast autocrine regulation in the control of bone remodeling. *Bone.* 2003;33:206-15.
25. Hruska KA, Mathew S, Saab G. Bone morphogenetic proteins in vascular calcification. *Circ Res.* 2005;97:105-14.
26. Li X, Yang HY, Giachelli CM. BMP-2 promotes phosphate uptake, phenotypic modulation, and calcification of human vascular smooth muscle cells. *Atherosclerosis.* 2008;199:271-7.
27. Dorman LJ, Tucci M, Benghuzzi H. In vitro effects of bmp-2, bmp-7, and bmp-13 on proliferation and differentiation of mouse mesenchymal stem cells. *Biomed Sci Instrum.* 2012;48:81-7.
28. Jeong JO, Han JW, Kim JM, Cho HJ, Park C, Lee N, Kim DW, Yoon YS. Malignant tumor formation after transplantation of short-term cultured bone marrow mesenchymal stem cells in experimental myocardial infarction and diabetic neuropathy. *Circ Res.* 2011;108:1340-7.
29. Yoon YS, Park JS, Tkebuchava T, Luedeman C, Losordo DW. Unexpected severe calcification after transplantation of bone marrow cells in acute myocardial infarction. *Circulation.* 2004;109:3154-7.
30. Liao J, Chen X, Li Y, Ge Z, Duan H, Zou Y, Ge J. Transfer of bone-marrow-derived mesenchymal stem cells influences vascular remodeling and calcification after balloon injury in hyperlipidemic rats. *J Biomed Biotechnol.* 2012;2012:165296.
31. Moreno PR, Purushothaman KR, Sirol M, Levy AP, Fuster V. Neovascularization in human atherosclerosis. *Circulation.* 2006;113:2245-52.